# Early divergent B cell differentiation during antibody responses

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# Abstract

The early B cell response to antigen is analyzed, probing the mechanisms of divergent differentiation into plasmablasts or germinal centre (GC) B cells. Heterozygous QMxB6 mice, in which 5% of B cells are specific for the hapten 4-hydroxy-3-nitrophenol (NP), were immunized with NP-Ficoll. Varying the antigen dose altered the proportion of B cells that entered the extra follicular or GC responses and the amount of class switch recombination (CSR). The expression of phenotypic markers and switched antibody protein in parallel with gene array analysis and single cell real time RT-PCR in responding B cells was used to identify GC and plasmablast precursors and when and where CSR occurred.

The results suggest CSR occurs in B blasts before GC B cells or plasmablasts emerge in both thymus dependent and thymus independent type II responses. The protein IRF4, which is essential for CSR and plasmablast differentiation, is expressed in all responding cells immediately after immunization and selectively upregulated to high levels in plasmablasts. A hierarchy of gene expression was identified as B cells differentiate into plasmablasts whereby high IRF4 mRNA expression precedes CD138 protein, which in turn precedes Blimp1 expression.

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# Abbreviations

| | |
|---|---|
| AFC | Antibody forming cell |
| AID | Acitvation induced cytidine deaminase |
| β2m | beta-2 microglobulin |
| B cell | Bursa of Fabricius cell |
| Bcl6 | B cell lymphoma 6 |
| BCR | B cell receptor |
| Blimp1 | B-lymphocyte-induced maturation protein 1 |
| Btk | Bruton tyrosine kinase |
| C57Bl6 | C57BL/6J |
| CDR | Complementarity determining region |
| CGG | Chicken gamma globulin |
| CSR | Class switch recombination |
| D | Diversity |
| DNA | Deoxyribonucleic acid |
| Ds | Double stranded |
| Fig | Figure |
| F | Follicle |

| | |
|---|---|
| FDC | Follicular dendritic cell |
| GC | Germinal Centre |
| Ig | Immunoglobulin |
| Int | Intermediate |
| IRF4 | Interferon regulatory factor 4 |
| J | Joining |
| κ | Kappa |
| λ | Lambda |
| LPS | Lipopolysaccharide |
| MZ | Marginal Zone |
| N | Nucleotide |
| NFκB | Nuclear factor of kappa light polypeptide gene enhancer in B-cells |
| NK | Natural Killer cell |
| NP | 4-Hydroxy-3-nitrophenylacetyl |
| Pax5 | Paired box gene 5 |
| Poly I:C | Polyinosinic:polycytidylic acid |
| QM | Quasi Monoclonal |
| QMxB6 | C57B6 mice heterozygous for the QM heavy chain (Section 2.1) |
| Rag | Recombination activating genes |

| | |
|---|---|
| RNA | Ribonucleic acid |
| RT2 PCR | Real-time reverse-transcriptase polymerase-chain-reaction |
| S | Switch |
| Ss | Single stranded |
| SHM | Somatic hyper mutation |
| STAT | Signal transducers and activators of transcription |
| T cell | Thymus cell |
| TCR | T cell receptor |
| T zone | T cell zone or Periarteriolar lymphoid sheath |
| TD | Thymus dependent |
| Tdt | Terminal deoxynucleotidyl transferase |
| TI | Thymus independent |
| UNG | Uracil-DNA glycosylase |
| V | Variable |
| XBP-1 | X-box binding protein 1 |

# 1. Introduction

This thesis is concerned with the differentiation of B cells as they respond to antigen; their location, gene expression at the protein and mRNA level and how this influences the outcome of the B cell response. B cells are effectors of the adaptive immune system through their production of antibody and activation factors. In this way B cells play an important role in the resolution of a wide range of infections. Antibody in the blood and tissue fluids acts in a wide range of ways: directly through neutralization of virus particles and toxins and indirectly by activating complement as well as a range of cellular effectors including macrophages, neutrophils, eosinophils, mast cells, platelets and natural killer (NK) cells.

There are several different types of B cells and these participate in different types of response. This introductory section will provide a brief didactic outline, without references, of topics covered later.

B lymphocytes are characterized by their expression of surface bound antibody. This, complexed with other associated proteins, forms the B cell receptor (BCR). Antibody is composed of two heavy chain molecules covalently bound to each other. Each is bound to a light chain molecule. During development B cells undergo rearrangement of the variable regions of their heavy and light chain immunoglobulin (Ig) genes, a process that endows each B cell with one of an almost limitless number of possible receptors. As this process can produce antibodies that are reactive to self antigens, elaborate mechanisms act which serve to eliminate, alter the BCR specificity or render such B cells inactive.

The expression of antibody on the BCR enables the specific recruitment of B cells into antibody responses. The simplest and most rapid form of antibody

response is the extrafollicular response. In these responses B cells are induced to proliferate as B blasts before differentiating into proliferating antibody-secreting cells – plasmablasts, and then into non-proliferating antibody secreting cells – plasma cells. All of the types of antigen discussed below can evoke extrafollicular responses. Antigens that induce antibody responses have been classified according to whether the responses they evoke are thymus dependent (TD) or thymus independent (TI). The latter can operate in congenitally athymic mice, which lack thymus-processed T cells. TD antigens, by contrast, require cognate interaction between thymus-processed T cells and B cells in order to proceed. Thymus indepedent responses have been suclassified into type 1 and 2 on the basis of whether they can occur in CBA/N mice which lack the X-linked Bruton tyrosine kinase. TI-1 antigens can evoke responses in this mouse, while TI-2 antigens cannot. All three types of response, TD, TI-1 and TI-2 are characterized by a bias in the types of B cells that respond and also in their outcome.

T cell independent type 2 responses (TI-2) are typically, but not exclusively, evoked by antigens that contain polysaccharide chains that contain repeating sequences. These are typified by the polysaccharides that form the capsules of pneumococci and meningococci. The repeating sequences in these antigens can cross link many BCRs on the same B cell producing a larger signal sufficient to induce a proliferative antibody response. By contrast TI-1 responses are induced by antigens that trigger innate signalling receptors on B cells as they activate the BCR. The most obvious example of this is the effect of the lipopolysaccharide (LPS) of the outer layer of the outer cell membrane of gram negative bacteria. Toll-like receptor 4 (TLR4) on B cells binds the invariant lipid A of LPS and, if the BCR recognises the

polysaccharide of the LPS moiety, which varies between different types of bacteria a response may be evoked.

T cell dependent antigens require specific activation of both T and B cells for a response to be generated. The activation of naïve T and B cells by antigen induces them to relocate into a common area of lymphoid tissue and acquire characteristics that enable them to interact with each other. T cell dependent antigens characteristically induce germinal centres (GC) in addition to extrafollicular responses. In GC B cells undergo proliferation and activate a process of Ig Variable (V)-region gene-directed hypermutation. This is followed by antigen and T cell-dependent selection; GCs can result in a substantial increase in the affinity of the antibody produced by their constituent B cells. A proportion of selected GC B cells differentiate into plasma cells that can be long-lived and some become memory B cells. Whilst basic scientific research tends to focus on T cell dependent or T cell independent antigen pathogens frequently present a variety of antigens and may invoke a range of responses. For example, salmonella produce a TD B cell response to flagellin and a TI response to outer membrane proteins. Similarly, sheep red blood cells in mice evoke both TD and TI responses.

Developmental and phenotypic features have been used to define two major subsets of B lymphocytes – B1 and B2 cells. Most naïve B cells in the secondary lymphoid tissues of adults are B2 cells and these have been subclassified into $IgM^{+}$,$IgD^{high}$,$CD23^{high}$,$CD21^{+}$, $B220^{high}$ recirculating B cells and $IgM^{+}$,$IgD^{low/-}$,$CD23^{low/-}$,$CD21^{high}$, $B220^{high}$ marginal zone (MZ) B cells. B1 cells are characteristically $IgM^{+}$,$IgD^{low/-}$,$CD23^{low/-}$,$CD21^{low}$, $B220^{int}$ and in mice they have been subclassified into B1a cells which express CD5 and B1b cells which are CD5 negative. Memory B cells from GC differ from naïve B2 cells in terms of their responsiveness and their

quantity. In addition, the participation of B1 cells in memory responses has recently been described. Neither B1 nor B2 memory will not be considered further at this stage.

TD responses may recruit B cells from the recirculating and MZ pools. It is less clear whether they can also recruit cells from the B1 compartment. TI-2 antigens typically induce a response in B1 cells as well as MZ B cells. By contrast recirculating B cells are not recruited into responses to TI-2 antigens. The recruitment of B cells into TI-1 responses depends at least in part upon whether they express Toll-like receptors and analogous receptors. B1 and MZ cells are more likely to express these receptors than recirculating B cells.

This study examines the differentiation of B cell blasts into early plasmablasts or GC cells in the spleen. Whilst this thesis is mostly concerned with the TI-2 response to NP-Ficoll the study begins the process of examining the role of key transcription factors in a TD response. This introduction will describe the structure of the spleen, the development of B cells and the B cell receptor (BCR). The different types of B cells that exist will then be described followed by a résumé of the response to antigen and the differentiation of B cells into GC and antibody producing cells.

### 1.2. The structure of the spleen.

My studies have focused exclusively on the mouse spleen. In essence the spleen is formed as a continuum of tissue perfused by blood sinusoids – the red pulp - in which islands of more compact tissue – the white pulp areas – are suspended. This organ has at least four groups of functions in the adult mouse. Firstly macrophages on the cords of Billroth within the red pulp blood sinusoids remove

from the blood pathogens and time-expired or opsonised blood cells (Schmidt, *et al*, 1988). Second the red pulp in mice, in contrast to that of humans, contains haemopoietic tissue (Johnson, *et al*, 1975). Third, the red pulp contains poorly-characterized niches that sustain plasma cell survival (Sze, *et al*, 2000). The fourth and most important splenic function in the context of this thesis relates to the secondary lymphoid tissue which is located in islands of the white pulp. This secondary lymphoid tissue hosts the B and T cell responses of the spleen. At the centre of each island of white pulp there is a central arteriole (Schmidt, *et al*, 1988), and surrounding this is the T zone, or PALS (perioarteriolar sheath) characterised by the presence of T cells and antigen-presenting dendritic cells (DC) (Fig 1.1). The T zone is cupped by B cell areas - follicles, which are in turn surrounded by the MZ. Small areas of the T zone between the follicles abut the red pulp directly; these are called bridging channels. Bridging channels are the point of exit of recirculating B and T cells from the white pulp (Bajenoff, *et al*, 2008) and also an exit point for plasmablasts generated in extrafollicular antibody responses that grow in extrafollicular foci in the adjacent red pulp (Jacob, *et al*, 1991b; Toellner, *et al*, 1996).

### *1.2.1. The T zone*

At the centre of each white pulp island lies the T Zone. Recirculating T cells leave the blood in the MZ blood sinusoids and the migrate into this area (Howard, *et al*, 1972) where they interact with the resident DC, testing their T cell receptor (TCR) against the antigen presented by the DC (Stoll, *et al*, 2002). T cells that have a TCR that recognizes antigen presented in the context of MHC class I or II molecules or CD1d form a synapse with the DC and start the process of T cell priming (Austyn, *et al*, 1988; Mempel, *et al*, 2004). After about 30 hours these T cells enter cell cycle and

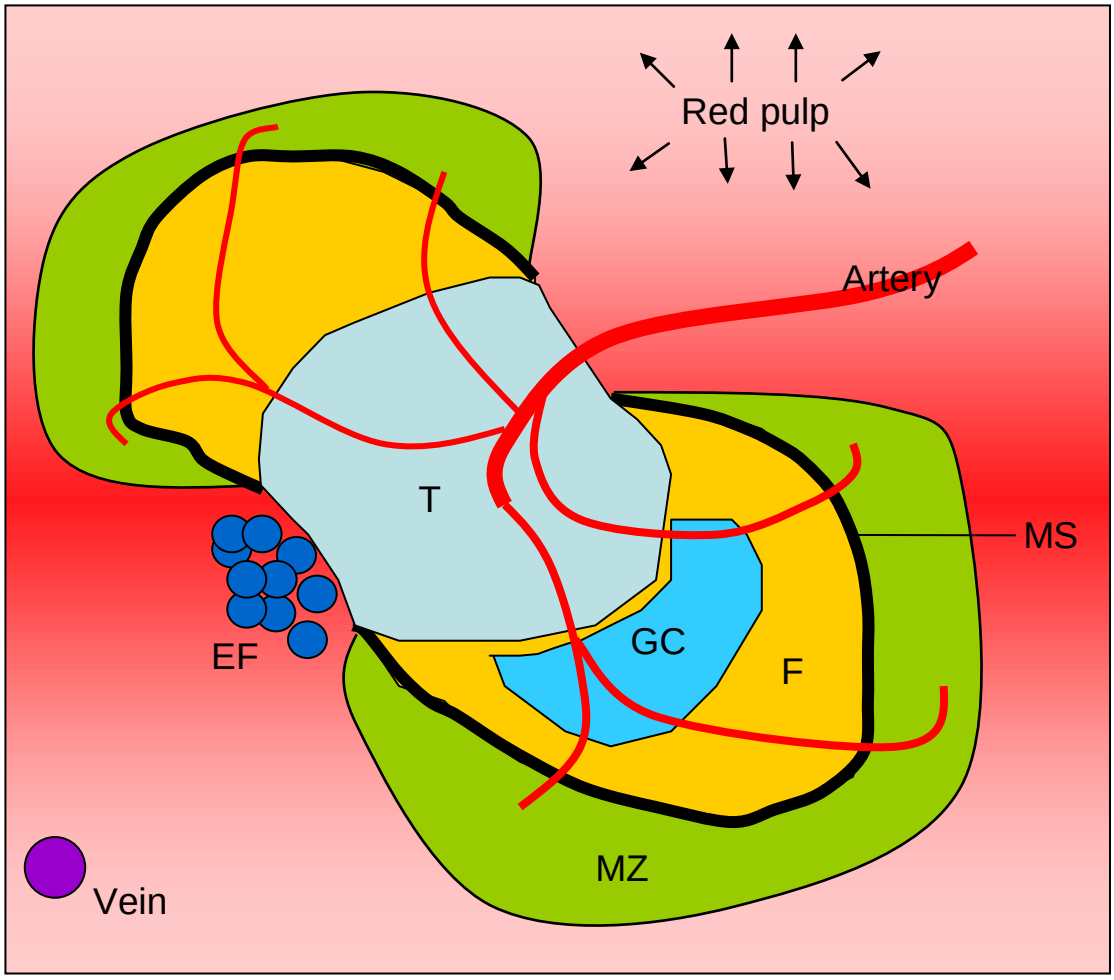


Figure 2.1 The structure of the white pulp of the spleen

The murine white pulp consists of a T cell area, the T zone (T), partially encapsulated by the B cell rich follicles (F). The follicles are separated from the marginal zone (MZ) by the marginal sinus (MS). Where the red pulp abuts the T zone extra follicular foci (EF) are formed at the bridging channels during early antibody responses to antigen. Germinal centres (GC) form in the follicle.

over the next 18 hours migrate to the boundary of the T zone and the follicle changing their expression of CCR7 and CXCR5 (Hardtke, *et al*, 2005). It is at the boundary of the T zone and follicle where they interact with B cells that have taken up and processed antigen via their BCR (Toellner, *et al*, 1996; Okada, *et al*, 2005). B cells are induced to migrate to the boundary of the T zone and the follicle regardless of whether the response is TI or TD (Okada, *et al*, 2005; Hsu, *et al*, 2004). Analysis of how B cells responding to TI antigen behave in the T zone is a major topic of this thesis.

*1.2.2. The follicles*

The follicles, B cell areas that partially surround the T zone, are predominantly composed of recirculating B cells along with follicular dendritic cells (FDC) (Tew, *et al*, 1997). When follicles contain no GC they are known as primary follicles. Secondary follicles contain GC and the recirculating cells form a rim, the follicular mantle, around the part of the GC that does not abut the T zone. Recirculating B cells are found throughout primary follicles and they are in a constant state of migration between the follicles of secondary lymphoid tissues (Niewenhuis and Ford, 1976). Characteristically, recirculating B cells are $IgD^{high}$, $IgM^{+}$, $CD23^{high}$, $CD21^{+}$, $B220^{high}$ (Gray, *et al*, 1984; Ling *et al*, 1987; Coffman and Weissman, 1986).

Follicular dendritic cells take up antigen from transporting B cells (Brown, *et al*, 1970; Phan, *et al*, 2007) and can retain this antigen for months (Tew and Mandel, 1978). During a TD response GC B cells with a corresponding BCR may take up antigen from FDC and present this to follicular T cells (Kosco, *et al*, 1988). Recently evidence has been presented that indicates recirculating B cells can take up antigen from FDC in the first few days following antigen administration (Suzuki, *et al*, 2009)

*1.2.3. The marginal zone*

The MZ is a band of cells in the spleen that surround that part of the B cell follicles that does not abut to the T zone. The MZ contains B cells (Kumararatne, *et al*, 1981), macrophages that can localize neutral polysaccharides (Humphrey, 1979: Humphrey and Grennan, 1981) and metallophilic macrophages. In rats and mice the entire marginal zone is perfused by blood sinusoids, while in humans only the outer portion has a sinusoidal blood supply. It is separated from the follicular areas by the marginal sinus – a thin band of MadCAM expressing cells that mark the inner limit of the MZ blood sinusoids (Kraal, *et al*, 1995). The cells in the MZ come into direct contact with the blood from the marginal sinuses (Schmidt, *et al*, 1988). MZ B cells are discussed in detail in section 1.4.2.1.

The marginal sinus is the point at which both recirculating T and B cells enter the spleen and T zone. They migrate to the edge of the follicles where they meet bridging channels (Howard, *et al*, 1972; Niewenhuis and Ford, 1976). The T cells then migrate to the DC in the central T zone while the B cells move to the follicles via the outer T zone (Niewenhuis and Ford,1976). If these T or B cells fail to encounter an antigen they recognise they leave the white pulp after about a day via MZ bridging channels (Nieuwenhuis and Ford,1976).

## 1.3. The development of B cells

The B cell compartments in mice are not fully formed at birth. B cells are produced in the foetal liver, the spleen from late foetal life and shortly after birth (particularly in the first 3 months although B lymphopoiesis can continue for longer in the spleen) (Johnson, *et al*, 1976). B lymphopoiesis occurs in the bone marrow from late foetal life onwards (Johnson, *et al*, 1976). In mouse and human the B cell subset

formed depends upon the stage of development, with the evidence from reconstitution experiments pointing to B1 lymphopoiesis being confined to the fetal liver, while B2 lymphopoiesis occurs mainly in the bone marrow. Recently evidence has been presented that indicates in mice B1a lymphopoiesis in post natal life is confined to the spleen, while B2 lymphopoiesis predominantly procedes in the bone marrow (Rosado, *et al*, 2009). Whilst debate persists around whether the different B cell subsets originate from a common precursor or from different development pathways, all B cells rearrange their B cell receptor (BCR). This section will describe first how the BCR is formed and then the development of the different B cell subsets.

### *1.3.1 The production of the B Cell Receptor*

Each B cell produces its own BCR (Raff, *et al*, 1973). This is produced through the rearrangement of the immunoglobulin heavy chain genes by a process of somatic recombination with imprecise splicing and, particularly in the bone marrow, the insertion of additional nucleotides (N) in the joining region. These processes can yield almost limitless Ig V region structures and hence a very extensive repertoire of BCR. The first recombination is between a diversity (D) segment, of which there are twenty five, and one of the six joining (J) segments in each Ig heavy chain allele (Alt, *et al*, 1984). This occurs simultaneously on both heavy chain gene sets (ten Boekel, *et al*, 1995). Secondly, this combined DJ region on one of the heavy chain gene complexes is joined to one of the variable region genes to produce the completed variable section of the BCR (ten Boekel, *et al*, 1995; Early, 1980). Should the rearrangement be unproductive then the other heavy chain will be rearranged (Alt, *et al*, 1984; Jung, *et al*, 2006). In addition to the permutations produced through the combination of V, D and J segments, nucleotides can be

inserted at the joins through the action of terminal deoxynucleotidyl transferase (Tdt) (Komori, *et al*, 1993). Mice deficient for Tdt have a restricted repertoire but homology-directed recombination increases variability (Gilfillan, *et al*, 1993). Terminal deoxynucleotidyl transferase is not expressed during development in the foetal liver (Li, *et al*, 1993) and this provides a bias in the type of BCR produced (Gilfillan, *et al*, 1993) e.g MZ B cells seeded from the immature B cell pool show a bias for cells that lack N insertions in the complementarity determining region (CDR) (Carey, *et al*, 2008).

This rearrangement of the heavy chain variable region genes is dependent upon the production of a recombinase complex involving both recombination activating genes (RAG) (Oettinger, *et al*, 1990) and 53BP (Difilippantonio, *et al*, 2008). A failure to produce a successful Ig heavy chain and light chain rearrangement, or in T cells a TCR with rearranged $\alpha\beta$ or $\gamma\delta$ chain genes, results in a lack of B and T cells. However, this can be overcome through the insertion of transgenic pre-arranged heavy and light chains (Reichman-Fried, *et al*, 1990).

The newly formed heavy chain is combined into the pre-BCR with surrogate light chain (SLC) and the BCR-associated transmembrane signalling molecules Ig$\alpha$ and $\beta$. This pre-BCR is expressed on the developing B cell (Lanig, *et al*, 2004; Keyna, *et al*, 1995). Signalling from a preBCR surrogate induces a reduction in Tdt, Rag1 and Rag2 expression and is independent of the SLC (Galler, *et al*, 2004; Wasserman, *et al*, 1997). Thus further re-arrangement is at least temporarily prevented. This pre-BCR signalling is dependent upon Ig$\alpha$ (Galler, *et al*, 2004) and oligomerization of the complex rather than antigen recognition (Bankovich, *et al*, 2007).

The expression of the rearranged heavy chain with the SLC induces a period of proliferation followed by the rearrangement of the light chain (Rolink, *et al*, 2000). Unlike the production of the heavy chain Tdt is not expressed during rearrangement of the light chain (Grawunder, *et al*, 1995; Wasserman, *et al*, 1997). In addition, the light chain rearrangement consists of only the J and V regions (Bernard, 1978). One kappa light chain is rearranged first, and if this is not successful the second kappa chain genes are rearranged. If neither can form a BCR with the heavy chain then the lambda chain genes are rearranged one after the other (Brauninger, *et al*, 2001).

On the production of a BCR, there are a number of outcomes for the B cell. Most newly-produced immature B cells leave the bone marrow and migrate via the blood to the T zone of the spleen (Lortan, *et al*, 1987). However, some immature B cells will have autoreactive BCR. These cells can be eliminated, become tolerant or anergic (Rolink, *et al*, 1998). At the newly-formed B cell stage BCR ligation can trigger receptor editing which may produce a non-auto-reactive BCR (Tiegs, *et al*, 1993). Whilst non reactive B cells tend to become recirculating B cells, some weakly autoreactive cells become MZ B cells or B1 cells (Chen, *et al*, 1997; Kanayama, *et al*, 2005; Xing, *et al*, 2009).

In un-manipulated conditions BrdU studies show that apoptosis occurs in 90% of immature B cells after 3-4 days in the periphery (Chan and Maclennan, 1993; Fulcher and Basten, 1997; Rolink, *et al*, 1998; Opstelten and Osmond, 1983). B2 cell survival and maturation is promoted through the expression of other factors such as B cell-activating factor (BAFF) (Schneider, *et al*, 2001, Schiemann, *et al*, 2001). There is evidence that newly produced B cells compete with recirculating B cells to gain access to the recirculating pool (MacLennan and Chan, 1993). The average lifespan of recirculating B cells is in the order of 6 weeks (MacLennan and Gray,

1986; Fulcher and Basten, 1997), indicating that mature recirculating B cells are only being replaced at a slow rate. Thus in steady state conditions immature B cells only have a small chance of displacing a recirculating B cell. On the other hand, if the recirculating B cell pool is depleted then immature B cell recruitment into the pool is massively increased (Bazin, *et al*, 1985). Conversely in the absence of immature B cell input recirculating B cell lifespan is extended indefinitely (Sprent, *et al*, 1991). Anergic autoreactive B cells fail to compete with non-autoreactive immature B cells for follicular niches (Cyster, *et al*, 1994). MZ B cells have a slightly shorter average lifespan than recirculating B cells, in the order of around a month (MacLennan and Grey, 1986; Hao and Rajewsky, *et al*, 2001).

### 1.4. B cell subsets and their roles

As mentioned above all B cells have their own BCR and can be induced to produce antibody given the correct stimuli. However, B cell subsets have been identified on the basis of their location and functional differences that reflect their different phenotypes. The main subdivision is between B2 cells, which include MZ and recirculating B cells, and B1 cells. B1 cells can be divided into $CD5^+$ B1a and $CD5^-$ B1b subsets.

#### *1.4.1. Recirculating follicular B cells*

The largest of the B cell populations in mature mice are recirculating follicular B cells. These can be identified through their phenotype - $IgD^{high,}$ $IgM^{int/low}$, $CD23^{high}$, $CD21^-$, $B220^{high}$ (Ling, *et al*, 1987; Grey, *et al*, 1984; Coffman and Weissman,1986). These develop in the bone marrow and as described above a small proportion are selected in the spleen either to join the recirculating pool of B cells or become MZ B

cells (Section 1.3.1). Once selected into the recirculating pool these cells enter a constant state of migration between follicles of the secondary lymphoid organs (Niewenhuis and Ford 1976). The process ceases only if the B cells engage antigen through their BCR and are recruited into an antibody response. Although it is likely that recirculating in the blood or lymph B cells can bind fluid-phase antigen there is evidence that specialized macrophages or DC can trap and present intact antigen to B cells (Balazs, *et al*, 2002; Wykes, *et al*, 1998). Recent data using in vivo imaging indicate that recirculating cells in the follicles can take up antigen from antigen-trapping macrophage-like cells located in subcapsular sinus of lymph nodes (Phan, *et al*, 2007; Carrasco and Batista, 2007). Also in the first few days after antigen administration recirculating B cells can pick up antigen that has been localized on the surface of FDC (Suzuki, *et al*, 2009).

1.4.1.1. The function of recirculating follicular B cells

Recirculating follicular B cells respond to T cell dependent antigen. In doing so they can be recruited to make an extrafollicular or follicular response or both of these (a brief description of the follicular response is found in section 1.5, extrafollicular responses are described in section 1.6). In addition recirculating follicular B cells may respond to Thymus independent type 1 (TI-1) antigens if they express the appropriate accessory receptors e.g. TLR4 will respond to bacterial outer cell wall lipopolysaccharide associated with many TI-1 antigens (Zhang, *et al*, 1988; Meyer-Bahlburg, *et al*, 2007). Conversely, recirculating follicular B cells do not appear to respond to TI-2 antigen (Lane, *et al*, 1986).

### *1.4.2. Marginal Zone B cells*

Marginal zone B cells have been characterised as being $IgM^{high}$ or switched

$Ig^{+}$, $IgD^{low/neg}$, $CD21^{high}$, $CD23^{int/low}$ (Ling, *et al*,1987; Grey, *et al*, 1984) and express CD35 (Molina, *et al*, 1996). Marginal Zone B cells are recruited through a number of processes. Firstly, directly from immature B cells (Carey, *et al*, 2008). There is also evidence from studies both in rats (Kumararatne and MacLennan, 1981; Bazin, *et al*, 1985) and mice (Vinuesa, *et al*, 2003) that recirculating follicular B cells can differentiate into MZ B cells by a proliferation and antigen-independent process. Memory B cells from GC will also enter the MZ in rats (Liu, *et al*, 1988; Liu, *et al*, 1991) and humans (Dunn-Walters, *et al*, 1995). Evidence for or against the presence of memory B cells in the proportionately much smaller MZ of mice is still inconclusive. Development of the MZ has been shown to be dependent upon Notch signalling (Song, *et al*, 2008). Indeed, mice heterozygous for Notch 2 show a reduction in B1a, B1b and MZ cells despite splenic structure remaining sound (Witt, *et al*, 2003). Marginal zone B cells are absent in mice that lack signalling through pyk-2, a tyrosine kinase which may compromise responses to chemokines and B cell adhesion (Guinamard, *et al*, 2000).

1.4.2.1. The function of marginal zone B cells

How MZ B cells behave upon engagement with antigen depends upon the ligand with which the antigen is bound. Marginal zone B cells that bind antigen with their BCR are induced to migrate to the boundary of the follicle and T zone and participate in the immune response (Liu, *et al*, 1988; Liu, *et al*, 1991). During the response to antigen MZ B cells can induce both early antibody production in extra follicular foci in responses to TD, TI-1 and TI-2 antigens (Liu, *et al*, 1991; Oliver, *et al*, 1999) or form productive GC (Song and Cerny, 2003). This process, and the subsequent onset of proliferation and differentiation in the T zone, will be considered in detail in the results section of this thesis.

Marginal zone B cells may also bind antigen, or immune complex, via their complement receptors and be induced to move into the follicle (Gray, *et al*, 1984). This migration is essential for immune complex transport to FDC in the spleen (Brown, *et al*, 1970; Gray, *et al*, 1884; Kroese, *et al*, 1986). Recently it has been shown that MZ B cells that transport antigen shuttle back and forth between the MZ and the follicle (Cinamon, *et al*, 2008). The movement of MZ B cells between the follicle and MZ is dependent upon the cyclical upregulation followed by down regulation of CXCR5 which enables movement towards CXCL13, produced in the follicles. This alternates with upregulation and downregulation of SIP-1 which senses $S1P_1$ – a chemokine present in the blood in the MZ sinusoids (Lu and Cyster, 2002; Cinamon, *et al*, 2004; Cinamon, *et al*, 2008). The strong signals delivered by LPS which was used by Gray, *et al* (1984) and Petterson, (1967) to induce MZ B cell migration, may well inhibit temporarily the expected $S1P_1$-driven migration back to the MZ, for the MZ is largely emptied for a day or so.

*1.4.3. The development of B1 cell subsets*

The two subset of B1 cells recognized in mice are distinguished by the expression of CD5 by B1a cells (Hayakawa, *et al*, 1984) and the lack of CD5 expression by B1b cells (Solvason, *et al*, 1991). Because of this marker B1a cells were extensively studied before B1b cells. Unlike B2 cells B1a and B1b cells are self replenishing (Lalor, *et al*, 1989; Hsu, *et al* 2006). Whether a B cell becomes a B1 or a B2 cell is dependent upon a number of factors that are not absolute:

1) The BCR expressed: – B1 and B2 cells show characteristic BCR and, if a mouse is engineered to express only one type, or the other, only B1 or B2 cells will result (Lam, *et al*, 1999)

2) The strength of signalling from the BCR and BCR associated proteins (Casola, *et al*, 2004; Haas, *et al*, 2005; Hayakawa, *et al*, 1986; Carvalho, *et al*, 2001)

3) The age of the mouse: - In mice B1 development occurs mainly in the foetal liver (Hayakawa and Hardy, 1985), spleen (Rosado, *et al*, 2009) and omentum (Solvason, *et al*, 1991). The primary production of B1a cells has mainly been associated with the foetal liver (Esplin, *et al*, 2009; Wortis and Berland, 2001), although the recent findings of Rosando *et al* suggest this may continue after birth in the spleen. B1b by contrast do not appear to be produced during foetal life and seem to be produced later during development (Hardy and Hayakawa, 1991; Kantor, *et al*, 1992).

There has also been a hunt to find markers for a proposed B1 precursor. Montecino-Rodriguez (2006) reported that a $CD45^{low\text{-}neg}$ $CD19^{+}$ population includes a B1 specific progenitor. This was followed by a report from Tung (2006) of $CD138^{+}$ and I-A denoting a B1a specific precursor cell in embryonic mice. Furthermore, transfer experiments have suggested that B1 cells can be derived from both early lymphoid and common lymphoid progenitors (Esplin, *et al*, 2009).

Recently there has been a trend to describe $CD11b^{-}$ peritoneal B1 cells as B1c (Malkiel, *et al*, 2009; Hastings, *et al*, 2006). Whether this is an accurate portrayal of B1 development is contested (Ghosn, *et al*, 2008). Complicating things yet further, there has also been some discussion as to the production of B1 cells in wild type mice and how the B1, subset should be defined (Thiriot, *et al*, 2007).

#### 1.4.3.1 The role of B1 cells

B1 cells are mostly found in the peritoneal cavity; however, a few B1a cells reside in the spleen (Hayakawa, *et al*, 1983; Hayakawa, *et al*, 1985) and lymph

nodes (Hsu, *et al*, 2006). There is relatively little known about the details of B1 cells responding to antigen. They can form plasmablasts that proliferate in extrafollicular foci in the spleen and they can give rise to plasma cells in the spleen (Hsu, *et al*, 2006) omentum (Ha, *et al*, 2006; Ryan, 2009) and lamina propria of the gut (Rosado, *et al*, 2009).

B1a and B1b cells have been shown to participate differently in response to antigens. B1a cells constitutively produce “natural antibody” (Baumgarth, *et al*, 1999) the amount of which regulates the cell numbers present (Baker and Ehrenstein, 2002). Natural antibody is the term given to that found in profoundly antigen-deprived mice, i.e. germ free mice fed on chemically defined diets and reared in stainless steel cages. (Bos, *et al*, 1989a and b). B1a cells can respond to LPS (Yang, *et al*, 2007), streptococcal polysaccharides (Wardemann, *et al*, 2002) and influenza (Choi and Baumgarth, 2008). B1b cells provide long lasting humoral T cell independent immunity to the model antigen NP-Ficoll (Hsu, *et al*, 2006; Obukhanych and Nussenweig, 2006) and respond to bacterial antigens such as *Borrelia hermsii* (Alugupalli, *et al*, 2004) and and are responsible for the early response to *Salmonella Typhimurium* (Gil-Cruz, *et al*, 2009).

### *1.4.4. Memory B cells*

Memory B cells provide early antibody against recurrent infection (Liu, *et al*, 1991; Song and Cerny, 2003). Their participation allows a secondary response to antigen to occur with greater speed than the primary infection (Toellner, *et al*, 1996). Memory B cells are classically produced by GC, and frequently but not always, express switched antibody isotypes (Coico, *et al*, 1983). Indicative of their GC origin they frequently contain mutations in their CDR (Dunn-Waters, *et al*, 1995; Anderson,

*et al*, 2007). In mice $CD80^+$ memory B cells are found in the blood, MZ and the follicle (Anderson, *et al*, 2007). In humans and rats the location of memory cells is less controversial than in mice. Memory cells have been identified in the rat and human MZ (Liu, *et al*, 1988; Dunn-Walters, *et al*, 1995) but they also reside in the bone marrow (Lausen, *et al*, 2004). CD27, a marker which is associated with switched and mutated cells (Klein, *et al*, 1998; Maurer, *et al*, 1992) is found on B cells in the blood (Agematsu, *et al*, 1997). However, the use of CD27 as a marker of human memory B cells, is questionable as memory B cells have been found in children with hyper IgM syndrome associated with CD40L deficiency. These children have very little switched antibody and probably do not produce GC suggesting not all memory cells are post GC (Weller, *et al*, 2004; Weller, *et al*, 2001).

Recent studies in mice have shown that in response to TI-2 antigen B1b cells can form long lived memory cells (section 1.4.3.1). As my thesis is concerned with the early response to antigen the production of memory cells is not investigated.

### 1.5. B cell responses to antigen

There are two pathways that a B cell may follow after antigen engagement. The B cell may proliferate and then differentiate into plasmablasts that produce antibody from three or so days after immunization. These plasmablasts proliferate and differentiate into plasma cells in extra follicular foci adjacent to the T zone (MacLennan, *et al*, 2003) Alternatively, after a short period of proliferation in T zones, the antigen-activated B cell may undergo an extended period proliferation in follicle centres, where they undergo somatic hyper mutation (SHM) of the variable regions of the antibody heavy and light chains (Jacob, *et al*, 1991a; Toellner, *et al*, 2002). This GC response is followed by antigen-and CD4 T cell driven selection which may

result in B cells producing a higher affinity antibody for the antigen (Berek, *et al*, 1985; Jacob, *et al*, 1991a). B cells that leave the GC go on to form antibody secreting plasma cells in the bone marrow (Blink, *et al*, 2005) or memory B cells (Coico, *et al*, 1983).

Whilst different types of B cells may have a greater predisposition to one pathway or the other it has been shown that in TD responses, and some TI responses in IgH knock-in mice, both extrafollicular and GC cells are likely to arise from the same subset of B cells with the same BCR (Jacob and Kelsoe, 1992; de Vinuesa, *et al*, 2000). It is still unclear if a single B cell can proliferate in response to antigen and give rise to some cells that form GC and others that become extrafollicular plasmablasts.

### *1.5.1. T cell independent B cell responses*

Experiments in neonatally thymectomised mice, which lack thymus-processed T cells, show that antibody can be produced in response to certain antigens in the absence of T cell help (Davies, *et al*, 1969; Davies, *et al*, 1970). These T cell independent (TI) responses are typically carried out by MZ and B1 cells (Balazs, *et al*, 2002; Vinuesa, *et al*, 2003; Hsu, *et al*, 2006). As already mentioned, T independent antigens can be divided into two types – TI-1 and TI-2. T cell independent type 2 antigens are defined by their inability to induce a productive antibody response in mice deficient in Bruton's tyrosine kinase (Amsbaugh, *et al*, 1972; Scher, *et al*, 1975a; Scher, *et al*, 1975). T cell independent type 1 antigens can evoke productive responses in the absence of this kinase. TI-1 antigens, such as LPS (Cohen, *et al*, 1976), when given in high doses can induce polyclonal B cell responses through TLR4 ligation. Much lower doses of LPS are required for specific

B cell activation that comes from LPS cross-linking TLR-4 with the BCR. Type 2 antigens induce a B cell response through cross linking of many BCR molecules on one B cell. They characteristically possess long carbohydrate or in some cases protein chains with repeating epitopes, e.g. NP-Ficoll (Cohen, *et al*, 1976).

Without T cell help GC are formed in only very exceptional circumstances, for example in the response of mice with an NP-specific transgenic BCR to high dose NP Ficoll (de Vinuesa, *et al*, 2000). These GC involute after 5 days, without producing plasma cells or memory B cells, probably as a result of failed selection of GC B cells, for these cells cannot process and present carbohydrate-based antigens to GC T cells. Ig variable region mutation does occur in these GC, but at a far lower rate than in TD GC (Toellner, *et al*, 2002).

### *1.5.2. Binding antigen induces B cells to migrate to the T zone follicular boundary*

Marginal zone and follicular B cells are induced to migrate to the T zone/follicular boundary once they have engaged antigen and express CXCR5 to allow travel into the follicle (Cinamon, *et al*, 2008; Okada, *et al*, 2005). B cells travel along the CCL19/21 gradient to the boundary of the T zone and expression of CCR7 maintains their position (Reif, *et al*, 2002; Okada, *et al*, 2005).

### *1.5.3. B cells receive differentiation signals at the boundary of the T zone and follicle*

In the T zone responding B cells are induced to proliferate as B blasts. They do not enter their first mitosis until around 24 hours after BCR ligation and then undergo some 3 cell divisions during the second day of the response (Hsu, 2004). It is at the boundary of the T zone and follicle that, in a T cell dependent response, B cells receive T cell help through making cognate interaction with primed CD4 T cells

(Okada, *et al*, 2005). This can occur within twelve hours of the B cells first engaging antigen (Toellner, *et al*, 1996). In TI responses B cells may receive signals from resident NK cells (Gao, *et al*, 2008) and DC (Wykes, *et al*, 1998; Litinskiy, *et al*, 2002) that induce class switch recombination (CSR) or differentiation towards forming antibody producing cells. This takes place through the action of TNF family molecules such as BAFF, which promotes B cell survival, and IL-10 (Xu, *et al*, 2008; Litinskiy, *et al*, 2002). After the period of proliferation in the T zone some B cells may differentiate into plasmablasts and migrate to extra follicular foci where they continue proliferating for some time before coming out of cell cycle and differentiating into mature plasma cells (Sze, *et al*, 2000; Section 1.6).

*1.5.4. The formation of germinal centres*

Germinal centres form in a specialised area of the follicle where the VDJ regions of participating B cells are mutated in a process called somatic hyper mutation (SHM) (Jacob, *et al*, 1991a; Toellner, *et al*, 2002). This random process may result in an increase in affinity for the antigen that induced the GC formation. On the other hand the affinity may fall or alter and in some cases become autoreactive. A selection process described below serves to select positively those cells that attain a higher affinity for the antigen that induced the GC, while resulting in the death of other GC B cells.

Germinal centres are oligoclonal being founded by as few as 3 B cells (Kroese, *et al*, 1987; Liu, *et al*, 1991; Jacob, *et al*, 1991b). GC are not a closed unit, for new GC-founding B cells can enter a GC after it has been formed and participate in the response (Vonderheide, *et al*, 1990; Schwickert, *et al*, 2007). In GC B cells undergo proliferation and mutation of their DNA in the dark zone before migrating to

the light zone where they undergo apoptosis within a few hours unless they can obtain positive selection signals. These signals result firstly from binding antigen through their BCR (Liu, *et al*, 1991) and secondly making cognate interaction with local CD4 T cells after processing and presenting this antigen (MacLennan, 1994). Those B cells that receive appropriate signals from T cells are induced to follow one of three pathways. They may leave the GC and form AFC that may find niches in the red pulp of the spleen, but more typically migrate to the bone marrow, where they can become long-lived plasma cells (Benner, *et al*, 1981). Secondly they may become memory B cells (Coico, *et al*, 1983), or revert to become GC blasts that undergo further proliferation, mutation and selection (MacLennan, *et al*, 1997; de Vinuesa, *et al*, 2000). B cells that do not have a BCR that can bind available antigen, or cannot make cognate interaction through their BCR undergo apoptosis in situ (Liu, *et al*, 1991; Kawabe, *et al*, 1994). Although, the above hypothetical model for B cell affinity maturation and selection in GC is compatible with available data it remains a model that requires further testing. For example, the understanding of the way GC B cells are selected to follow one of at least 3 differentiation pathways is at best only partially understood. More recently, mathematical modelling coupled to *in vivo* work investigated the likely rate-limiting steps in GC reactions and the forces that drive affinity maturation (Meyer-Hermann, *et al*, 2009).

B cells in GC are characterized by the expression of the protein B cell lymphoma 6 (Bcl6) (Catoretti, *et al*, 1995) whilst the processes of SHM and CSR in GC are dependant upon the expression of activation-induced cytidine deaminase (AID) and a variety of other co-factors (Cascalho, *et al*, 1998). As Bcl6 and AID are used for markers of GC cell fate in my study they are now discussed in greater detail (Sections 1.5.4.1 and 1.5.4.2 respectively).

1.5.4.1 Expression of Bcl6 and the formation of germinal centres

B cell lymphoma-associated molecule 6 was first identified as a protein encoded by a gene that was dysregulated by translocation in certain B cell lymphomas (Baron, *et al*, 1993). The expression of Bcl6 protein is predominantly associated with B cells in GC (Cattoretti, *et al*, 1995). Further evidence for the crucial role of Bcl6 has been provided by the transfer of $Bcl6^{-/-}$ B cells which can not differentiate into GC (Fukuda, *et al*, 1997). The continued expression of Bcl6 counters differentiation of B blast in to an antibody producing cell (Shaffer, *et al*, 2004; Phan, *et al*, 2005). This occurs through the repression of B-lymphocyte-induced maturation protein 1 (Blimp1) (Reljic, *et al*, 2000; Tunyaplin, *et al*, 2004), X-box binding protein (XBP-1) and other genes involved in guiding differentiation towards plasmablasts and plasma cells. Bcl6 expression has been shown to be enhanced through the proteins Signal transduction and transcriptional activation (STAT) 3 and STAT 5 as well as Activator protein 1 (Scheeren, *et al*, 2005; Arguni, *et al*, 2005).

In order for a B cell to differentiate into an antibody producing cell Bcl6 must be downregulated (Catoretti, *et al*, 2005). It has been reported that this occurs through the expression of Blimp1 (Shaffer, *et al*, 2002) and Interferon regulatory factor 4 (IRF4) (Saito, *et al*, 2007).

*1.5.5. Class switch recombination*

The efficacy and function of antibody can be modified in two ways. Firstly the variable region of the antibody can be altered so as to provide a higher affinity for the antigen and, as described above, this occurs in GC (section 1.5.4). Secondly, the class or subclass of the antibody produced can be changed and consequently its

functionality may be altered – for example, IgA which is predominantly secreted in the gut, or IgE which is secreted mainly in the tissues at the exposed internal or external surfaces of the body, i.e. the skin as well as the respiratory and intestinal tracts (Rabbits, *et al*, 1980; Cory, *et al*, 1980).The process by which the isotype of the antibody produced is changed is called class switch recombination (CSR). In the mouse, B cells can undergo CSR of the rearranged V region genes to any one of the downstream heavy chain genes. These are located in order from the $\mu$ and $\delta$ genes: - $C\gamma_3$, $IC\gamma_1$, $C\gamma_{2a}$ (or $C\gamma_{2c}$ in C57B6 mice), $C\gamma_{2b}$, $C\varepsilon$ and $C\alpha$. The isotype to which the cell switches depends upon the stimulus provided to the cell. For example, TI-2 antigens characteristically induce switching to $IgG_3$ (Garcia de Vinuesa, *et al*, 1999), whereas T cell dependent antigens that evoke Th2 responses induce switching to $IgG_1$, while those causing Th1 differentiation typically result in switching to IgG2a and IgG2b (Toellner, *et al* ,1998). Switching patterns can be modified in a range of different ways by a variety of innate immune signals and cytokines (Le Bon, *et al*, 2001).

Class switch recombination occurs in GC (Liu, *et al*, 1996; Pape, *et al*, 2003), prior to the extrafollicular response (Garcia de Vinuesa, *et al*, 1999), in mucosal sites (Takhar, *et al*, 2005) and in vitro (Muramatsu, *et al*, 2000). As such, whilst it can be induced through BCR stimulation alone it can also be triggered by signalling from other cells and through other receptors on the B cell (Gao, *et al*, 2008; Xu, *et al*, 2008; Liu, *et al*, 1991; Jacob, *et al*, 1991b).

1.5.5.1. The mechanism of CSR

Class switch recombination occurs through the juxtaposition of the VDJ region of the BCR with the immunoglobulin $C_H$ gene to which the cell is to switch. The

process proceeds through a number of stages. Firstly, transcription through the switch (S) region in both the μ locus and the destination locus (Kataoka, *et al*, 1980; Nambu, *et al*, 2003; Shinkura, *et al*, 2003). These germline switch transcripts are processed removing the introns (Hein, *et al*, 1998) and are essential but not sufficient for the process of CSR (Lorenz, *et al*, 1995). Class switch recombination is dependent upon the action of the enzyme AID on single stranded DNA (ssDNA) (Yu, *et al*, 2005). It has been postulated that these areas of ssDNA are induced during transcription of the switch region through hybridisation of the germline DNA with the RNA produced (Yu, *et al*, 2003). These R loops provides access to the DNA for AID and uracil-DNA glycosylase (UNG) and nicks in the exposed strand to be made (Yu, *et al*, 2003; Yu, *et al*, 2005; Huang, *et al*, 2007). There is further discussion of the function and role of AID in the following section.

In the next stage DNA in between the target switch region and the VDJ is excised through the formation of a switch circle (Matsuoka, *et al*, 1990; von Schwedler, *et al*, 1990). The ends are joined through the nonhomologous end joining pathway. Once recombination has occurred switched transcripts are produced and the antibody secreted by or expressed on the surface of the cell will be of new isotype.

#### 1.5.5.2 Activation Induced Cytidine Deaminase

Activation Induced Cytidine Deaminase is an enzyme that removes the amine group from cytidine in single stranded DNA (Petersen-Marht, *et al*, 2002). This process leaves behind a uracil:guanine mismatch in the DNA which may be repaired through excision by the enzyme UNG (Schrader, *et al*, 2005; Petersen-Marht, *et al*,

2002). This produces single stranded breaks in the DNA where recombination or DNA repair can occur (Faili, *et al*, 2002; Petersen-Marht *et al*, 2002).

The importance of AID has been elucidated through a combination of both *in vitro* and *in vivo* experiments. In AID knock-out animals neither CSR nor SHM occur (Nagaoka, *et al*, 2002; Muramatsu, *et al*, 2000). By removing the DNA repair mechnisms (UNG and MutS homolog 2) it has been shown that AID induces mutations along S regions of the immunoglobulin loci prior to CSR (Xue, at al, 2006). The AID dependent excisions occur on both DNA stands (Schrader, *et al*, 2005; Xue, *et al*, 2006) and if two are close enough together a double stranded break can result (Chaudhuri, *et al*, 2003; Rush, *et al*, 2004). A complex containing AID (Li, *et al*, 2006) is targeted to the switch regions through a number of processes- histone acetylation, transcription of switch transcripts as well as the sequence of the DNA itself (Nambu, *et al*, 2003; Wang, *et al*, 2006; Rogozin and Diaz, 2004).

Because of it's ability to produce mismatches during transcription and thus increase the mutation rate in a cell when it is present in the nucleus the cellular location of AID protein is very important (Ramiro, *et al*, 2003; Mcbride, *et al*, 2004). Visualisation of AID in the human tonsil has shown that it is strongly expressed in the cytoplasm of GC cells and in a small proportion of their nuclei (Muramatsu, *et al*, 1999; Cattoretti, *et al*, 2006). In addition, it has been reported that the exit of AID from the nucleus is an active process via a nuclear exit signal whilst it has been suggested that entry into the nucleus is a passive process. This restricts the time that AID can have access to DNA (Brar, *et al*, 2004; Mcbride, *et al*, 2004; Ito, *et al*, 2004). The phosphorylation state of AID has also been shown to be important as it allows interaction with replication protein A, a single stranded DNA binding protein (Basu, *et al*, 2005). However it has been reported that phosphorylation, whilst

increasing the speed and efficiency of CSR, is not essential for this process (Mcbride, *et al*, 2008).

Not surprisingly, the expression of AID is tightly regulated. Most recently the association of miRNA 155 has been shown to downregulate AID expression (Teng, *et al*, 2008; Rodriguez, *et al*, 2007) whilst miRNA 181b has also been shown to inhibit AID expression and subsequently CSR to γ1 (de Yebenes, *et al*, 2008). The 5' promoter region of the AID sequence has been shown to be bound by both Nuclear factor of kappa light polypeptide gene enhancer in B-cells (NF-κB) and STAT6 allowing CSR to be induced after CD40 or IL-4 stimulation respectively (Dedeoglu, *et al*, 2004). It must be mentioned that whilst the action of AID to deaminate cytidine has been widely reported and inferred there are some reports of AID independent double stranded DNA breaks (Zan and Casali, 2008).

### 1.6. Plasmablast and extrafollicular foci development

As discussed, B cells are induced to form plasmablasts either directly from the T zone in TI responses, or after a period of mutation and cell division in the GC (Liu, *et al*, 1991; Jacob, *et al*, 1992). Plasmablasts in extrafollicular responses divide rapidly for around three days before differentiating further into antibody producing plasma cells (Sze, *et al*, 2000). In the spleen available evidence is consistent with the extended survival of plasmablasts and plasma cells being dependent upon the limited stroma that supports these cells. Those cells that do not gain access to this stroma and consequently fail to receive sufficient survival signals undergo apoptosis (Sze, at al, 2000).

In order for a B cell to differentiate into a plasmablast it has to instigate a genetic programme involving the key regulatory genes Blimp1, IRF4 and XBP1 (Shapiro-Shelef, *et al*, 2003; Shaffer, *et al*, 2004; Soro, *et al*, 1999; Klein, *et al*, 2006). In my study plasmablasts were identified through their expression of CD138 with intermediate (int) or low levels of B220 (Smith, *et al*, 1996). Because of their role in the differentiation of B cells to plasmablasts and plasma cells Blimp1 and IRF4 are used as key markers of differentiation in this study.

*1.6.1. Blimp1*

B-lymphocyte induced maturation protein 1 is a transcription factor that is essential for the differentiation of B cells into antibody secreting cells (Soro, *et al*, 1999; Shapiro-Shelef, *et al*, 2003). It is necessary and sufficient for plasmablast differentiation (Shaffer, *et al*, 2002). In an analogous but reciprocal way to Bcl6, Blimp1 acts as a terminal repressor; it inhibits a variety of genes that are involved in preventing differentiation and increasing proliferation, such as myelocytomatosis oncogene (c-Myc) and Paired box gene 5 (Pax5) and Bcl6 (Lin, *et al*, 1997; Nera, *et al*, 2006; Shaffer, *et al*, 2004; Shaffer, *et al*, 2002). Through the inhibition of Pax-5 Blimp1 also downregulates expression of the BCR associated proteins CD79$\alpha$, CD79$\beta$ and CD19 (Hagman, *et al*, 2000; Nutt, *et al*, 1997). Besides acting as a repressor it is also responsible for the up-regulation of a number of genes that promote differentiation into an antibody secreting cell, such as XBP-1 (Shaffer, *et al*, 2004). Because of its important role in the terminal differentiation of B cells Blimp1 is used in these studies as a marker of terminal differentiation into an antibody producing cell.

*1.6.2. Interferon regulatory factor 4 and plasma cell development*

Interferon regulatory factor 4 (IRF4) knock out mice have been shown to develop normally until 4-5 weeks at which point they lose serum IgM levels and no antibody responses are produced (Mittrucker, *et al*, 1997). Expression of IRF4 has been reported to be induced through IgM cross linking (Matsuyama, *et al*, 1995). Conditional knock out mice have shown that plasma cell development from both GC and extrafollicular pathways is dependant upon IRF4 (Klein, *et al*, 2006). Indeed IRF4 is reported to be expressed at high levels in those plasma cells and B cells that, it has been inferred, are about to leave the GC (Falini, *et al*, 2000). The process of CSR is also inhibited in the absence of IRF4 (Klein, *et al*, 2006). Consequently it has been suggested that the level of IRF4 protein in the responding B cell may determine the induction of CSR at low levels or differentiation at higher levels (Sciammas, *et al*, 2006). The expression of IRF4 in GC-derived B cell lines, regardless of whether Bcl6 is being expressed, induces upregulation of Blimp1 and downregulation of Bcl6 and Pax5 (Teng, *et al*, 2007). Interferon regulatory factor 4 protein has been shown to be directly upstream of both Bcl6 and Blimp1, at least in the context of human CD32 gene expression (Gupta, *et al*, 1999; Gupta, *et al*, 2001). This indicates that IRF4 is a key protein in the B cell response and an ideal candidate for study in the induction of CSR and the regulation of GC and plasmablast formation.

## 1.7. B cell responses to 4-hydroxy-3-nitrophenol (NP) conjugates

The studies presented here utilise the well reported Quasi-monoclonal (QM) mouse system in combination with hapten 4-hydroxy-3-nitrophenol (NP) conjugated to either a long chain polysaccharide or a protein carrier. The epitope NP conjugated

to a variety of carriers has been used for a number of decades in mouse studies to induce immune responses (Bothwell, *et al*, 1981). It is predictable - the antibody response induced has a bias towards λ light chain and the Ig heavy chain V segment V168 (Bothwell, *et al*, 1981). Because of this restricted repertoire it is an ideal hapten for studies of SHM (Bothwell, *et al*, 1981; Blier and Bothwell, 1988). By changing the conjugate to which NP is bound the type of response induced can be varied, for example, the conjugation of chicken gamma globulin (CGG) (or another protein) to NP induces a TD response (Azuma, *et al*, 1986). However, the use of a polysaccharide such as Ficoll provokes a TI-2 type response (Maizels, *et al*, 1988).

### *1.7.1 The Response to NP-Ficoll*

The C57B6 mouse response to NP-Ficoll is characterised by a strong extrafollicular plasmablast response (Garcia de Vinuesa, *et al*, 1999). Twenty four hours after immunisation NP specific B cells are located in the outer T zone and by 48 hours these cells have produced germline switch transcripts to $IgG_3$ (Garcia de Vinuesa, *et al*, 1999). Plasmablasts are located in extrafollicular foci by 72 hours and these may persist there for months (Hsu, *et al*, 2006). These long-lived foci contain both switched and non switched antibody producing plasma cells (Maizles, *et al*, 1988). During the first 48 hours of this response the majority of NP-binding B blasts are located in the T zone but some are found in the follicle, in addition some plasmablasts may also be found in the red pulp (Garcia de Vinuesa, *et al*, 1999). The NP-binding blasts in the follicles reflect the inability of the responding cells to form GC. It has also been shown that B1b cells will respond to NP-Ficoll and as well as giving rise to extrafollicular plasmablasts memory cells induced that are seen mainly in the follicle (Hsu, *et al*, 2006). It seems likely that the sustained plasmablast

response to NP-Ficoll is mainly attributable to B1b cells, which like MZ cells, respond first by proliferating in the T zone and then producting plasmablasts in the extrafollicular foci (Hsu, *et al*, 2006). Unlike the B2 response, repeat exposure to antigen does not increase the affinity of B1b cells to the antigen (Azuma, *et al*, 1986).

*1.7.2. Quasi monoclonal mice*

Quasi Monoclonal mice were devised to have a heavy chain specific for the hapten NP when combined with a lambda light chain. The J chain sequences were replaced with a fully rearranged heavy chain (VDJ 17.2.25) identified in an NP-specific hybridoma (Cascalho, *et al*, 1996). The BCR only recognises NP when there is a productive λ light chain rearrangement. Consequently the original QM mouse which was designed to be monoclonal also had inactivated κ light chain genes. As implied by the name some editing of the transgenic heavy chain occurs so, even in a homozygous mouse on a kappa light chain deficient background, only around 60% of the B cells are NP-specific. There is evidence that this editing occurs in heptamer sequences in the IgV region (Cascalho, *et al*, 1997; Bertrand, *et al*, 1998; Han, *et al*, 1997). This is presumably as a result of not removing the endogenous V and D sequences before inserting the complete BCR sequence.

In the present study QM x C57B6 F1 hybrid mice have been used. These mice have one functional set of kappa chain genes. As the QM heavy chain rarely has NP-specificity when paired with an endogenously rearranged κ light chain only around 5% of B cells are NP-specific (Vinuesa, *et al*, 2001).

## 1.8. Aims of the project

This project was designed to study the differentiation of NP-specific B cells as they diverge to form either GC or antibody forming cells with particular reference to Ig class switching. By using immunohistochemistry, to locate cells throughout the response, in combination with flow cytometry to sort B cell populations as they differentiate it was possible to perform PCR on single and bulk sorted cells. This provided a means to define, at the genetic level, when and where cells differentiate. The behaviour of B cells was tested in both TI and TD contexts to identify consistent model for the B cell response to antigen in the spleen.

# Chapter 2: Methods and materials

## 2.1. Animals

QMXB6 mice contain one copy of the NP-specific (VH17.2.25-DSP2.3-JH4) Ig heavy chain segment of QM mice. These were generated by crossing QM mice (Cascalho, *et al*, 1996) to C57BL/6J for 10 generations, selecting for the NP-specific heavy chain. QMxB6 and C57BL/6J mice were maintained in the Biomedical Services Unit, University of Birmingham under specific pathogen free conditions. To test for NP specific BCR and the kappa light chain, the following primers were used:

### *2.1.1. Primers and concentrations for genotyping PCR*

| **Primer** | **Sequence** |
|---|---|
| β-actinF | GTGGGGCGCTCTAGGCACCA |
| β-actinR | CGGTTGGCCTTAGGGTTCAGGGGGG |
| NPF | TTCAGAGGTTCAGCTGCAGCAGT |
| NPR | CTYACCTGAGGAGACDGTGA |
| Jh-F | TCGCCTTCTATCGCCTTCTT |
| Jh-R | GCCCCAACTATCCCTCCA |
| JhWtF | AGCTCCCATACTTCATGGCCA |
| JhWTR | TTGGTCCTGAAGGCCCAGAAT |
| kappa-F | CTGGTGCTTTACGGTATCGC |
| kappa-R | GTAGAAGGCTCAAGGTTGTG |
| kappaWtF | TGCTGCACCAACTGTATCCA |
| kappaWTR | CAGGACGCCATTTTGTCGT |

## 2.2 Immunizations

T cell independent responses were induced through immunization with 30µg 4-hydroxy-3-nitrophenol (NP)- Ficoll (Biosearch, Santa Cruz, USA) in 200µl of sterile phosphate buffered saline (PBS) (pH 7.4) in to the peritoneum (*i.p.*). For early time points 30µg NP-fluorescein-Ficoll in 200µl PBS was used. Increased class switching was induced by co-immunizing with 30µg NP-Ficoll and 10µg Polyinosinic–polycytidylic acid (polyI:C) (Sigma Aldrich, Dorset, UK) in 200µl PBS *i.p.*.

A T cell dependant response was induced in C57Bl/6J mice as follows. Chicken gamma globulin (CGG) (Jackson Laboratories, Suffolk, UK) was precipitated in alum (aluminium potassium sulphate (Sigma Aldrich, Dorset, UK)). Briefly, 150µl CGG (1mg/ml) was mixed with 150µl 9% alum and the pH was adjusted to 6.5 using 10M sodium hydroxide. The solution was mixed at room temperature in the dark for 30 minutes before three washes in excess sterile PBS. Mice were immunized with 50µg CGG and $10^5$ heat inactivated Bordetella Pertusis i.p. (LEE laboratories, Georgia, USA). Five weeks after immunization mice were complemented with $5x10^5 – 2x10^6$ NP positive splenocytes *i.v.* 24 hours after transfer mice were immunized with NP-CGG *i.p*.

**2.3 Extraction of cells ex vivo**

Single cell suspensions of splenocytes were obtained by maceration of the spleen in 10% foetal calf serum (FCS, Invitrogen, Paisley, UK) and 1% penicillin/streptomycin (Invitrogen) supplemented RPMI media (Invitrogen) and filtration through a 70µm nylon mesh.

**2.4. In vitro cell culture**

Single cell suspensions of splenocytes were prepared as before and at all times were kept on ice (except for during incubation periods). Cells were treated with cyclohexamide at 2.5µg/ml (Calbiochem). Subsequent to this treatment they were stained and washed in cyclohexamide supplemented medium. Cells were plated at $2x10^6$ cells per ml in 96 well plates. NP-Ficoll was used to stimulate cells at a concentration of 1µg/ml.

## 2.5. Flow cytometry

Immunostaining of single-cell splenocyte suspensions took place at 4$^{o}$C for 20 minutes in a 1%FCS PBS buffer. Optimized concentrations of the following antibodies B220-FITC (eBioscience), CD138-biotin (BD Biosciences), and NP-phycoerythrin (Biosearch Technologies), followed by streptavidin-APC (BD Biosciences) were used following NP-Ficoll immunization. After NP-fluorescein-Ficoll immunization cell suspensions were incubated with B220-APC (BD Biosciences) and NP-phycoerythrin. Intracellular staining was obtained using the Cytofix/Cytoperm kit (BD Biosciences) according to the manufacturers' instructions, anti mouse IRF4 (Santa Cruz Biotech, CA, USA) and goat anti mouse Cy5 (Jackson Laboratories). Samples were acquired on a FACSCalibur (BD Bioscience) and were analyzed with FlowJo software (TreeStar, Oregon, USA). Cell sorting was done on a MoFlo cell sorter (DakoCytomation). Post sort populations were checked for purity and immediately frozen at -80$^{o}$C.

## 2.6. Histology

Tissues were snap frozen in liquid nitrogen and stored at –80$^{o}$C until required. Prior to sectioning tissue was mounted in OCT compound and quickly frozen. 6µm sections were cut using a cryostat (Bright Instruments, Huntingdon, UK) on to multispot slides (Hendley Essex Ltd, Loughton). Sections were left to air dry for one hour before fixation for 20 minutes in 4$^{o}$C acetone and stored at –20$^{o}$C. For RNA and protein analysis 20µm sections were cut and immediately frozen in liquid nitrogen.

Immunohistochemisty was carried out as described in Toellner, *et al*, (1996). In brief, slides were brought up to room temperature and hydrated in tris pH7.6 buffer (for composition see Appendix ii). Sections were subsequently kept in tris buffer at all

times. Primary antibodies were added at the optimum dilution for one hour and the slides were washed. Secondary antibodies were applied for 45 minutes (section 2.6.1 and 2.6.2). If required streptavidin complex with alkaline phosphatase was added (Dako, Glostrup, Denmark), after further washing, for 30 minutes.

Peroxidase and phosphotase reagents were developed sequentially (see appendix for buffer and substrate composition). Photographs for spleen sections were taken using a Leica CTR6000 microscope (Leica, MiltonKeynes, UK) and QCapture software. Images were processed using ImageJ (http://rsbweb.nih.gov/ij/).

*2.6.1 Primary antibodies*

| **Target** | **Isotype** | **Dilution** | **Manufacturer** |
|---|---|---|---|
| CD3 | Rt anti Ms | 1/2000 | BD Biosciences |
| IgD | Shp anti Ms | 1/300 | The Binding Site, Birmingham |
| IgM | Rt anti Ms | 1/600 | Serotech, Oxford, UK |
| NP | NP conjugated Rb | 1/2500 | Prof. P. Lane, Birmingham |
| Syndecan-1 | Rat anti Ms | 1/200 | BD Biosciences |
| IgD | Rat anti Ms | 1/1000 | BD Biosciences |
| IgG1 | Rat anti Ms | 1/400 | Serotech |
| IgG3 | Rat anti Ms | 1/300 | Serotech |
| Bcl-6 | Rb anti Ms | 1/30 | Santa Cruz Biotechnology, CA |
| IRF4 | Gt anti Ms | 1/400 | Santa Cruz Biotechnology, CA |
| CGG | Biotin | 1/500 | Prof P. Lane |

*2.6.2 Secondary and tertiary reagents*

| **Target** | **Dilution** | **Manufacturer** |
|---|---|---|
| Rb anti Rat biotin | 1/600 | Dako |
| Rb anti Rat PX | 1/50 | Dako |
| Dnky anti Shp biotin | 1/100 | The Binding Site |
| Dnky anti Shp PX | 1/100 | The Binding Site |
| Goat anti Hamster Ig [H+L] | 1/50 | Vector Labs, Peterborough, UK |
| Shp anti Rt biotin | 1/400 | The Binding Site |
| Swine anti Rb IgGs | 1/100 | Dako |
| Swine anti Rb biotin | 1/400 | Dako |
| Rabbit PAP | 1/100 | Dako |

## 2.7 Real time RT-PCR

RNA was extracted from cell pellets using the RNeasy Mini or Micro kits (Qiagen, Crawley, UK), eluted in water and reverse transcribed using 1μg of oligo-dT12-18 (Amersham Pharmacia Biotech, High Chalfont, UK) and Moloney murine leukemia virus reverse transcriptase (Invitrogen, Paisley, UK). Relative quantitation of specific cDNA species to β-actin message was carried out in a multiplex PCR on the ABI 7900 real time PCR machine (Applied Biosystems, Warrington, UK) as described (Cunningham *et al*, 2002). To detect the occurrence of Ig class switching to IgG3 recombined Iμ-γ3 recombined heavy chain transcripts were detected by real-time PCR. As Iμ is expressed constitutively (Li, *et al*, 1994; Lennon, and Perry, 1985) Ig class switch recombination from IgM to IgG3 should immediately be followed by transcription of recombined Iμ-γ3 heavy chain transcripts. Primers were in the first exon of IgM germline heavy chain RNA (TCTGGACCTCTCCGAAACCA) and the reverse primer (ACCGAGGATCCAGATGTGTCA, MWG Biotech, Ebersberg, Germany) and a probe in the first exon of the IgG3 heavy chain (FAM-CTGTCTATCCCTTGGTCCCTGGCTGC-BHQ1, Eurogentec, Seraing, Belgium). This was amplified in multiplex with β2-microglobulin as a housekeeping gene control with intron-spanning primers (CATACGCCTGCAGAGTTAAGCA, ATCACATGTCTCGATCCCAGTAGA, MWG Biotech) and a Yakima-Yellow Black Hole Quencher 1 labeled probe (CAGTATGGCCGAGCCCAAGACCG, Eurogentec). Relative quantification of signal per cell was done as described (Cunningham, *et al*, 2002). Thresholds within the logarithmic phase of the PCR for Iμ-γ3 recombined heavy chain transcripts and β2-microglobulin were set and the cycle number at which the threshold was reached (Ct) was determined using SDS 2.2.2 software

(Applied Biosystems). Ct for β-actin was subtracted from the Ct for Iμ-γ3 recombined heavy chain transcript. The relative amount was calculated as 2–ΔCt.

### 2.8 PCR Arrays

TaqMan low-density real-time PCR arrays (Applied Biosystems) were designed in a 96-gene format. Genes were chosen that were reported to be differentially expressed during early plasmablast or germinal centre cell differentiation (Shapiro-Shelef and Calame, 2004; Underhill, *et al*, 2003; Tarte, *et al*, 2003; Klein, *et al*, 2003). These are listed in the appendix (Appendix ii). RNA was isolated as above and mixed with QuantiTect Probe PCR Kit (Qiagen). This was added to the TaqMan Low-Density Array, and PCR was performed in a 7900HT Real-Time PCR System (Applied Biosystems) according to manufacturer's recommendations. Array data was analyzed using TMEV (Saeed, *et al*, 2003; http://www.tm4.org) and Applied Biosystems SDS 2.2.2 software. Relative signal per cell was calculated as above with β2-microglobulin as the house keeping gene control.

### 2.9 Single cell PCR

Using an automatic cell cloning unit of a MoFlo cell sorter single cells were sorted into wells of 384-well PCR plates containing 1μl nuclease-free water. Serial dilutions of 2 to 32 cells per well served as positive controls, one row of wells without cells as negative controls. After sorting plates were stored at -80ºC. Blimp-1, AID, BCL-6, IRF4, or β2-microglobulin mRNA were detected by triplex real-time RT2-PCR.

*2.9.1. Primers and concentrations for single cell RT2-PCR*

| Primer | Sequence | Concentration |
|---|---|---|
| Blimp-1F | CAAGAATGCCAACAGGAAGTATTTT | 80 nM |
| Blimp-1R | CCATCAATGAAGTGGTGGAACTC | 100 nM |
| AIDF | GTCCGGCTAACCAGACAACTTC | 60 nM |
| AIDR | GCTTTCAAAATCCCAACATACGA | 100 nM |
| BCL-6F | CAGACGCACAGTGACAAACCA | 60 nM |
| BCL-6R | ACTGCGCTCCACAAATGTTACA | 300 nM |
| β2-mglobF | CTGCAGAGTTAAGCATGCCAGTAT | 100 nM |
| β2-mglobR | ATCACATGTCTCGATCCCAGTAGA | 100 nM |
| IRF4F | GGAGGACGCTGCCCTCTT | 40nM |
| IRF4R | TCTGGCTTGTCGATCCCTTCT | 200nM |

*2.9.2 Probes for single cell RT2 PCR*

| Probe | Reporter/ Quencher | Sequence (Concentration) |
|---|---|---|
| Blimp-1 | FAM/MGB | TCTCTGGAATAGATCCGCCA (125nM) |
| AID | TET/BHQ | TGCATCTCGCAAGTCATCGACTTCGT (200nM) |
| BCL-6 | FAM/BHQ | CAGCCACAAGACTGTCCACACGGGT (100nM) |
| BCL-6 | CalFlour 560/BHQ | CAGCCACAAGACTGTCCACACGGGT (250nM) |
| β2-mglob | NED/MGB | CGAGCCCAAGACC (125nM) |
| IRF4 | FAM/BHQ | AGGCTTGGGCATTGTTTAAAGGCAAGTTC (150nM) |

Primers and probes were added to the lysate together with QuantiTect Multiplex RT-PCR buffer (Qiagen) in a total volume of 4µl. Reactions were run for 45 cycles in a 7900 Real-Time PCR System (Applied Biosystems). Relative quantification for gene expression in single cells was achieved by plotting the Ct from wells that showed a β2-microglobulin product in reverse order, corresponding to a log2 scale.

## 2.10 Quantification of cells following histology

In order to attain a representative figure for the area of the spleen occupied by $NP^+$ plasmablasts or $NP^+$ germinal centres (GC) sections of spleen were stained for NP and IgD. Using a graticule the number of intercepts that fell on $NP^+$ GC, as well as the total number of intercepts that covered the spleen, were counted and from this the percentage of grid intercepts occupied by $NP^+$ GC could be calculated. Similarly, the number of grid intercepts that fell on cells with $NP^+$ cytoplasmic staining was counted and the percentage of intercepts occupied calculated.

The number of $NP^+$ switched cells per $mm^2$ of spleen section was obtained through counting double positive (black) cells when sections were stained for both NP binding and the immunoglobulin isotype of interest (e.g. $IgG_3$). The area of the spleen section was calculated through the use of a graticule – the length of the side of the grid at each magnification was obtained using a haemocytometer.

In order to count the associations between $CD3^+$ cells and $NP^+$ cells sections were stained so as to have $CD3^+$ cells in blue and $NP^+$ cells brown. The number of $NP^+$ cells in contact and not in contact with a $CD3^+$ cell were counted for each spleen section.

## 2.11 Cell proliferation calculations

In order to calculate the number of cell divisions that responding B cells had undergone after NP-Ficoll immunization the number of $NP^+$ B cells prior to, and 48 hours after immunization was calculated (Table 2.1).

$$\text{Number of } NP^+ \text{ B cells in spleen} = \frac{\text{lymphocyte count}}{100} \times \%\text{lymphocytes } NP^+ B220^+$$

If we assume that QM B cells undergo 3 cell divisions after NP-Ficoll by 48 hours as deduced by Hsu (2004) it is possible to calculate the number of cells that must have responded to immunization, and, in the case of the response induced by 1μg NP-Ficoll, the number of cells that did not respond.

| | $NP^+ B220^+ CD138^-$ | | $NP^+ B220^+ CD138^+$ | |
|---|---|---|---|---|
| Time | 1μg | 30μg | 1μg | 30μg |
| 0 hours | $7.28x10^5$ | | $6.31x10^4$ | |
| 48 hours | $3.99x10^6$ | $5.74x10^6$ | $9.20x10^4$ | $2.47x10^5$ |

Table 2.1 The number of $NP^+$ B cells at the stated time after immunization with NP-Ficoll

The number of $NP^+ B220^+$ cells that are $CD138^+$ or $CD138^-$ prior to immunization and at 48 and 96 hours after immunization with 1μg or 30μg NP-Ficoll. Numbers were calculated from FACS of the samples and cell counts (Section 4. 1.1).

Number of cells that enter the response = $\frac{\text{Number of cells at 48 hours}}{2^3}$

For the 30μg response: $\frac{5.74x10^6}{8}$ = **$7.17x10^5$** (≈ $NP^+ B220^+$ cells at 0 hours)

For the 1μg response: $\frac{3.99x10^6}{8}$ = **$4.99x10^5$**

Therefore the number of $NP^+ B220^+$ cells that do not respond in the 1μg response is:

$7.28x10^5 – 4.99\ x10^5$ = **$2.29x10^5$**

So, if it is assumed that these cells do not proliferate after 1μg NP-Ficoll a proportion of cells at 48 hours do not participate in the response. This can be calculated thusly:

$\frac{\text{Number of non responding } NP^+ \text{ B cells}}{\text{Number of } NP^+ \text{ B cells at 48 hours}}$ x100

$\frac{2.29x10^5}{3.99x10^6}$ x 100 = **5.74%**

As CD138 becomes an unreliable marker of plasmablasts as they mature the FACS data at 96 hours is less reliable than the counts from histology (Table 2.2). Whilst the proportion of $NP^+$ plasmablasts was similar after 1μg or 30μg NP-Ficoll at 96 hours there is almost a five fold difference between the proportion observed.

| | % spleen area occupied by $NP^+$ plasmablasts | |
|---|---|---|
| Time | 1μg | 30μg |
| 0 hours | 1.32 | |
| 48 hours | 7.88 | 7.42 |
| 96 hours | 4.07 | 18.92 |

Table 2.2 The percentage of spleen area occupied by $NP^+$ plasmablasts at the stated time after immunization with NP-Ficoll

The percentage of grid intercepts that landed on $NP^+$ plasmablasts (as assessed by cytoplasmic NP staining) prior to immunization and at 48 and 96 hours after immunization with 1μg or 30μg NP-Ficoll.

To calculate the number of cells that continue in the 1μg response after 48 hours, and hence the number of cells that do not, we can consider the difference in the proportion of spleen sections occupied by $NP^+$ plasmablasts. Whilst these are equivalent at 48 hours by 96 hours the $NP^+$ plasmablasts occupy 5 times more of the spleen area in the 30μg NP-Ficoll response than in the 1μg response. If we assume that these are formed by precursors from the B blasts at 48 hours (at which point there are 1.4 times as many in the 30μg NP-Ficoll response than in the 1μg response), and that all the B blasts at 48 hours in the 1μg response should go on to form plasmablasts, then, given the five-fold difference between the two responses by 96 hours 70% of the B blasts did not.

B blasts at 48 hours: Number of 30μg B blasts = 1.44(Number of 1μg B blasts)

Plasmablast area at 96 hours: 30μg Plasmablasts = 5(1μg Plasmablasts)

Therefore, proportion of cells that did not expand: 1-(1.44/5) = .712

### 2.13 Quantification of B blast location at 48 hours in the carrier primed response

To ascertain whether there was a genuine difference in the location of B blasts at 48 hours after cell transfer in the carrier primed response to either NP-CGG or NP-Ficoll sections of spleen that had been stained for NP in blue and IgD in brown were photographed using QCapture software at 10x magnification with overlaps between each photograph. The resulting images were then aligned in power point before being printed out and stuck together (this produced an image approximately $1m^2$). From the top left of each spleen the follicle areas (as defined by brown staining) were halved and the number of $NP^+$ cells falling either side counted (Fig 2.1). The ratio of cells on the inner side of the follicle/ the outer side was taken for each. These values were added to with each successive follicle until the cumulative ratio stabilised (Fig 2.2). These final values were then used to produce the graph in Fig 5.5.

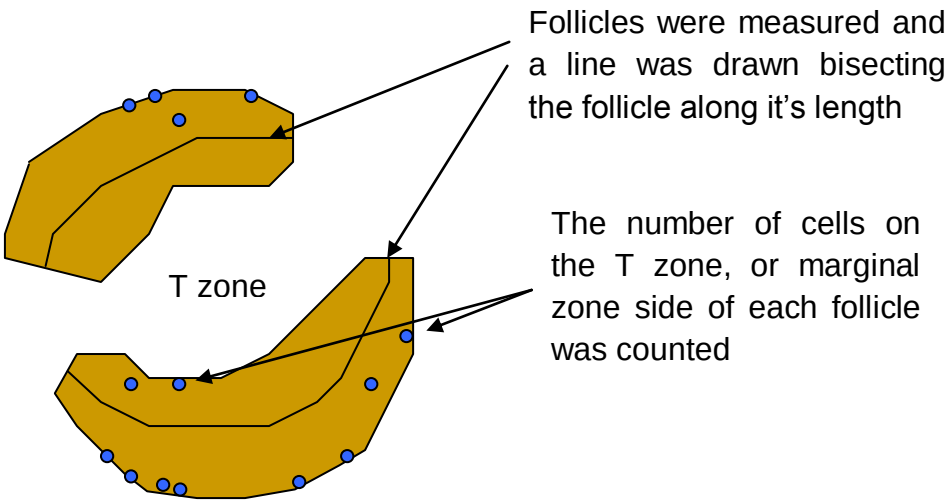


Figure 2.1: Counting the location of $NP^+$ B blasts at 48 hours in the carrier primed response

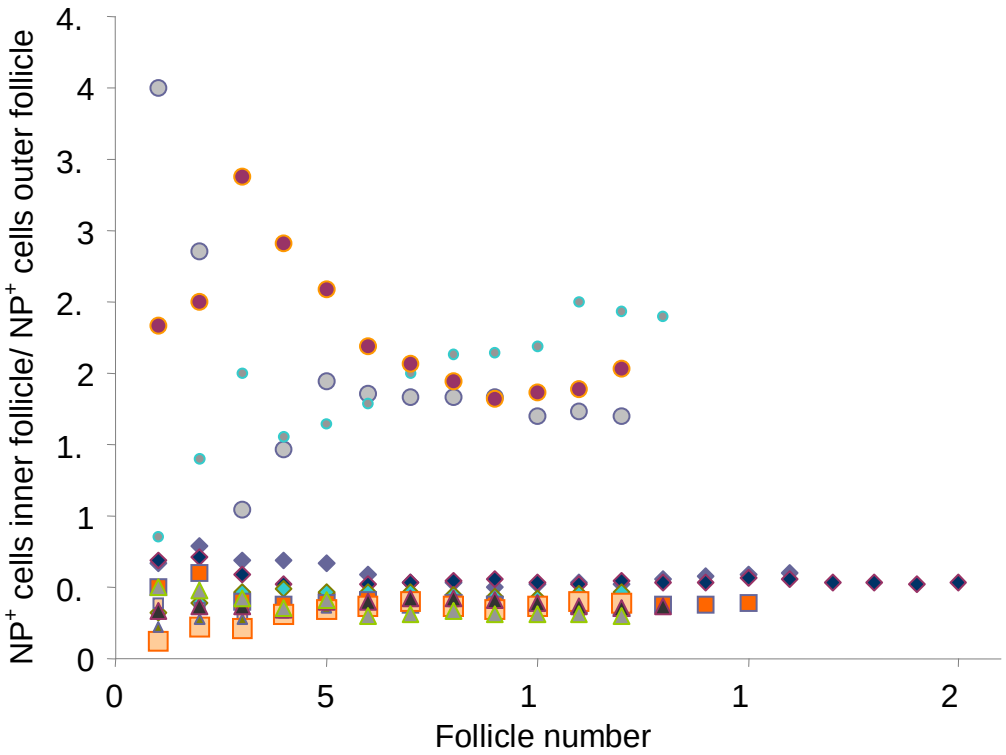


Figure 2.2: The cumulative ratio of $NP^+$ cells in the inner/outer follicle at 48 hours during the carrier primed response

# Chapter 3:

# Divergent B cell differentiation induced by 30μg NP-Ficoll

This project aims to study the divergent differentiation of B cells to germinal centre (GC) cells or extrafollicular plasmablasts during the response to antigen. The approach used histology, FACS and RT2 PCR (real-time reverse-transcriptase polymerase-chain-reaction) to identify the location and phenotype of the responding cells, the stages in extrafollicular and follicular B cell responses and gain insights into the mechanisms driving divergent differentiation.

*QMxB6 mice as an experimental model*

5-10% of the B cell complement in QMxB6 mice is specific for NP (section 1.7.2). After immunization *i.p.* with 30μg of the T cell-independent type 2 (TI-2) antigen NP-Ficoll these B cells respond to form either germinal centres or extrafollicular AFCs (de Viunesa, *et al,* 2000).

This study sought to identify and characterise GC and antibody former cells (AFC) precursors early in the response and ascertain when B cells commit to either developmental pathway. Additionally it was anticipated that the location of class switch recombination (CSR) in the extrafollicular response would be revealed. This protocol provided a means to investigate these questions.

*Experimental design*

Spleens of mice were examined 0, 24, 48 and 72 hours after immunization. Part of each spleen was snap frozen for immunohistology and the remainder was used to produce a single cell suspension. Splenocytes were used for flow cytometric

analysis. Phenotypically distinct B cell subsets were sorted either in bulk or as single cells for RT2-PCR analysis.

### 3.1. Immunohistological analysis of the early response to 30 μg NP-Ficoll

*3.1.1. Activated NP binding B cells move from the MZ to the T zone of the spleen within 8 hours of immunization*

Prior to immunization NP binding cells are located in the marginal zone (MZ) of the white pulp and a few background NP-specific plasma cells can be seen in the red pulp (Fig 3.1). Eight hours after immunization the MZ is depleted and NP binding cells appear at the boundary of the T zone and follicle. A dramatic sign of early phenotypic change is that the NP-specific cells, which lack detectable IRF4 protein before immunization, are clearly IRF4-protein-expressing by within 8 hours and this will be considered further in section 3.5.

*3.1.2. The NP-specific B cells become blasts in the T zone and remain in the white pulp throughout the 2$^{nd}$ day after immunization.*

It has been reported that responding B cells enter S phase within 24 hours of immunization (Garcia de Vinuesa, *et al*, 1999). They complete their first division shortly after this and have undergone some 6 cell divisions by 72 hours (Hsu, 2004). The B blasts remain mainly in the T zone although by 48 hours this becomes full and NP-specific B blasts are also found in the MZ, possibly reflecting overspill from the T zone (Fig. 3.2A). Although there are some NP-specific cells in the follicles at this stage they do not form a continuum of blasts in the follicle centre and, as described later, B cells

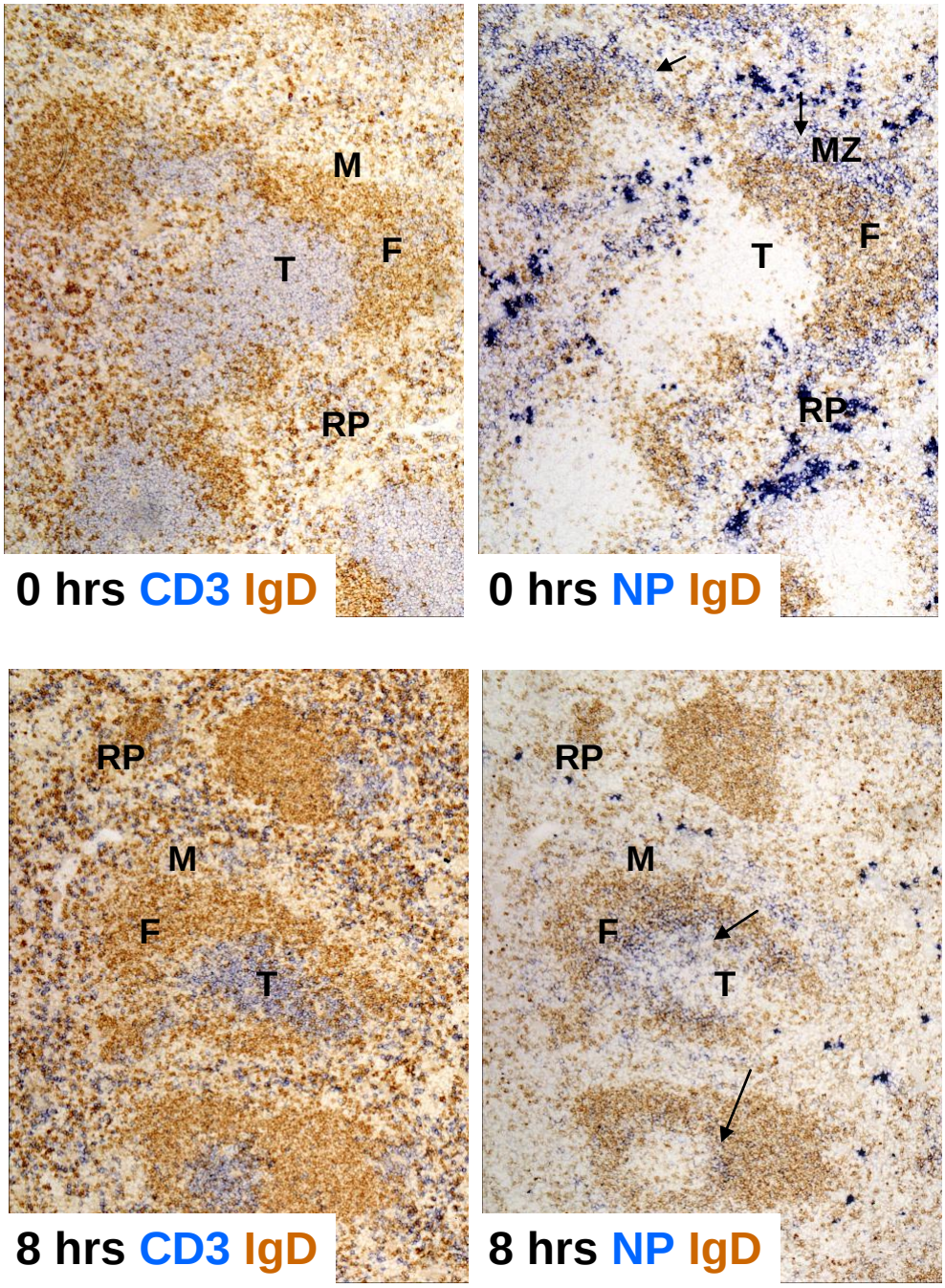


Figure 3.1 The early redistribution of NP specific cells in response to NP-Ficoll

At 0 hours the NP-specific cells stained blue (top right) and arrowed are located in the marginal zones (MZ). There are small numbers of dark blue AFC in the red pulp. By 8hrs the MZ are no longer filled with NP-specific cells and these are now seen in the outer T zone (arrowed in bottom right). The left hand figures highlight the follicles (F) containing IgD staining B cells (brown) and T zones (T) with $CD3^+$ cells (blue).

with a GC phenotype have not appeared. This is confirmed by the lack of Bcl6 expression in the follicular, or any other, NP-specific blasts at this stage (not shown). Bcl6 protein expression characterizes GC B blasts (Cattoretti, *et al*, 1995, Onizuka, *et al*, 1995). Strikingly at 48 hours there has been very little migration of NP-specific blasts out of the white pulp to the red pulp, although variable but always small, numbers of plasmablasts were seen in the red pulp at 48 hours (Fig 3.2A). This is shown objectively later in the chapter.

*3.1.3. Germinal centres and abundant AFCs appear on the 3$^{rd}$ day.*

On the third day after immunization GCs develop in the follicles of the white pulp (Fig. 3.2B). These are populated with NP-specific cells that express Bcl6 protein, with which they are associated (Fig. 3.3). As expected these cells lack detectable IRF4 protein, in contrast to the remaining T zone B blasts, which are still IRF4$^{+}$ and the now abundant of NP-specific AFCs in the red pulp and extra follicular foci. The plasmablasts are CD138$^{+}$, IRF4$^{high}$, Ki67$^{+}$ with cytoplasmic NP-binding (IRF4 expression will be addressed further in section 3.5).

## 3.2. NP binding B cells responding to NP-Ficoll divided into subsets according to their B220 and CD138 expression.

Flow cytometry was used to identify phenotypic changes in the responding populations of B cells. B220, a B cell associated protein (Coffman, *et al,* 1981) and CD138 (syndecan-1), expressed by plasmablasts (Sanderson, *et al,* 1989), were the key molecules studied in conjunction with NP binding.

Prior to immunization NP binding cells express high levels of B220 and bind high levels of NP. Twenty-four hours after immunization B220 expression is

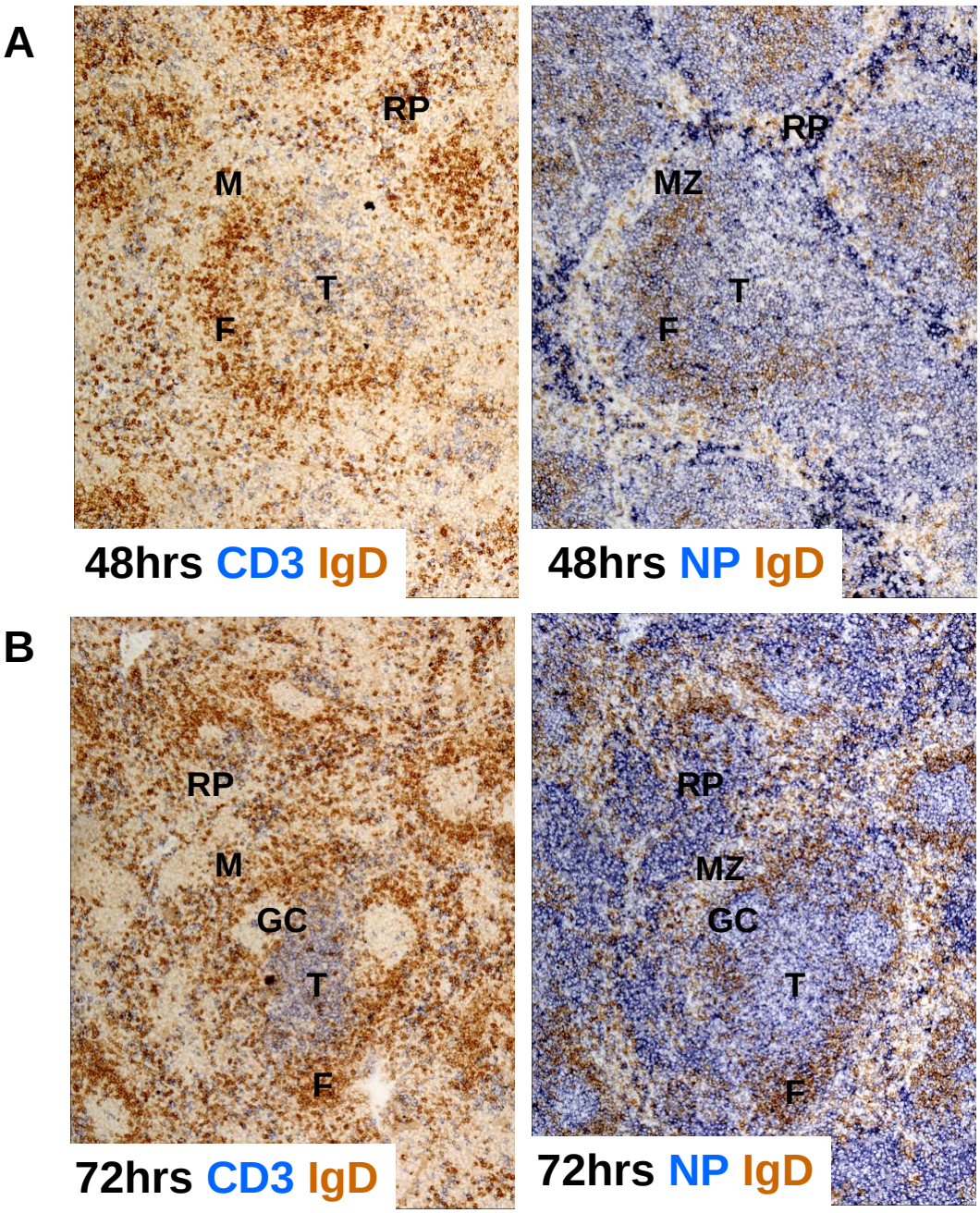


Figure 3.2: The expansion of NP specific cells in the splenic white pulp during the second day after immunization.

**A**. The NP-binding population has increased by 48 hours to fill the T zone. There are some plasmablasts in the red pulp but few compared to 72 hours. B. Germinal centres and plasmablasts are prominent at 72 hours. NP-binding cells re-appear in the MZ. IgD in brown and CD3 or NP in blue. GC= Germinal Centre, F= Follicle, T = T zone/ PALS, MZ = Marginal zone.

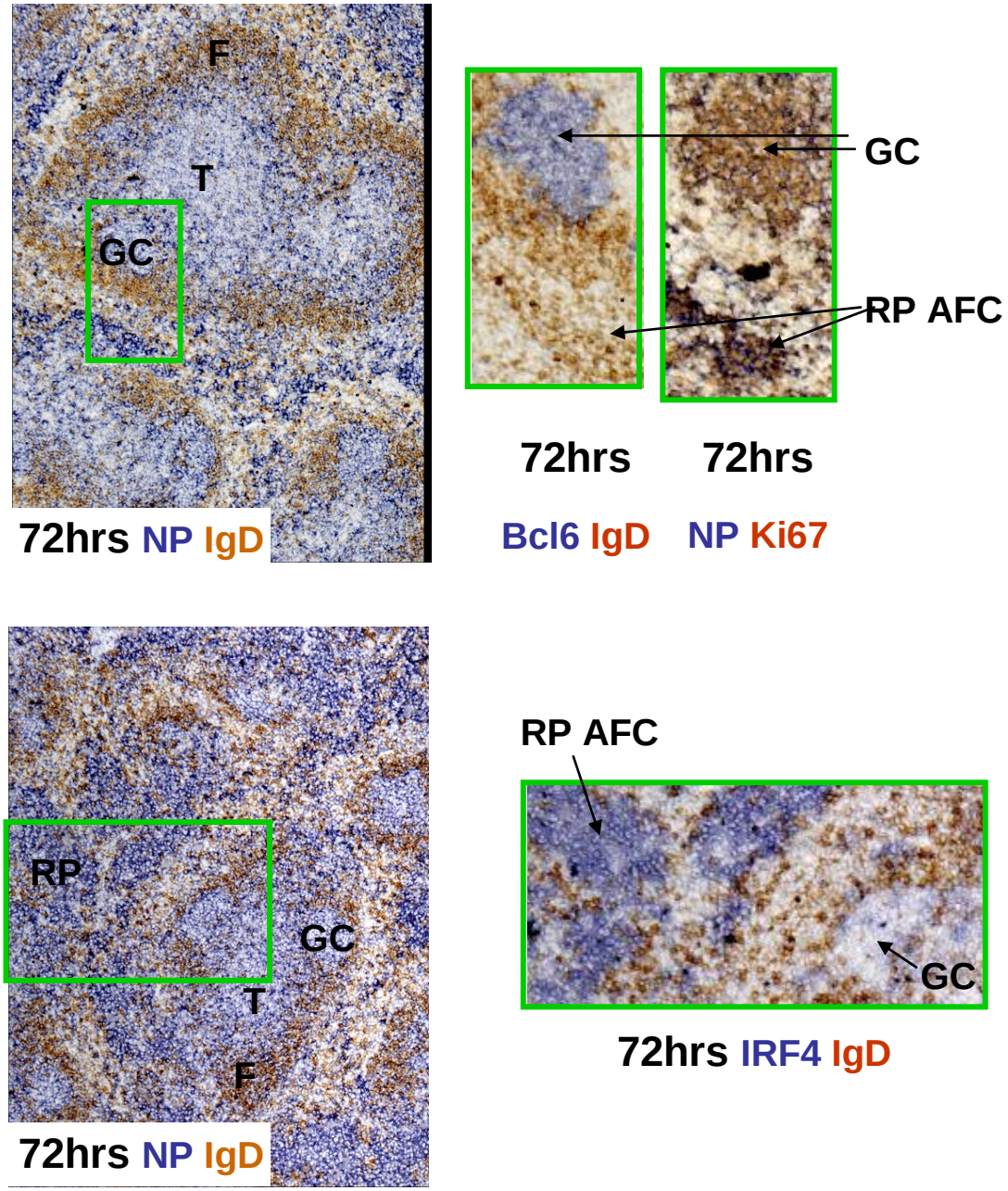


Figure 3.3: Germinal centre and plasmablast development in the QMxB6 response to NP-Ficoll

$NP^+$ germinal centres at 72 hours are Bcl6 and Ki67 positive. Antibody forming cells are $IRF4^+$. IgD or Ki67 in brown and CD3, NP or Bcl6 or IRF4 in blue. GC= Germinal Centre, F= Follicle, T = T zone/ PALS.

unchanged whilst the amount of NP bound has diminished considerably (Fig. 3.4). This change in NP binding may be due to competition from the NP-Ficoll with which the mice were immunized and perhaps BCR internalization. For 8 and 24 hour time points 30μg of fluorescent NP-FITC-Ficoll, which induced the same response as NP-Ficoll, was used to provide clear identification of responding cells. During the first 24 hours of the response the number of NP-binding cells remains the same (Fig 3.4C) consistent with the long $G_1$ observed by Hsu (2004).

B220 expression declines in NP binding cells as the B cells begin to differentiate at 48 hours. At this time the B220 intermediate (int) population represents ~30% of the NP binding cells. In agreement with the immunohistological data (Fig 3.2) a few $CD138^+$ B220 intermediate, plasmablast, cells are seen. During the second day the number of NP-binding cells increases more than 10 fold (Fig 3.4C). For this to occur all the responding cells at 24 hours need to have undergone cell division – this is again consistent with the CFSE studies of Hsu (2004). All three $NP^+$ populations - the B220 high, $B220^{int}$ and $CD138^+$ $B220^{int}$ - persist at 72 hours. However the $CD138^+$ $B220^{int}$ population now consists of 10-20% of the responding cells (Fig 3.4B) and the $CD138^-$ intermediate population makes up approximately 50%. In contrast to the previous day the number of $NP^+$ cells between 48 and 72 hours increases 2 fold (Fig 3.4C).

Bulk sorts were made of these populations to allow the mRNA expression profiles to be assessed in the responding cells during different stages of the response. These consisted of the $NP^+$ $B220^+$ population prior to and 24 hours after immunisation, the NP+ $B220^{high}$ and $NP^+$ $B220^{int}$ populations at 48 and 72 hours, and the $NP^+$ $CD138^+$ $B220^{int}$ population at 72 hours as previously described.

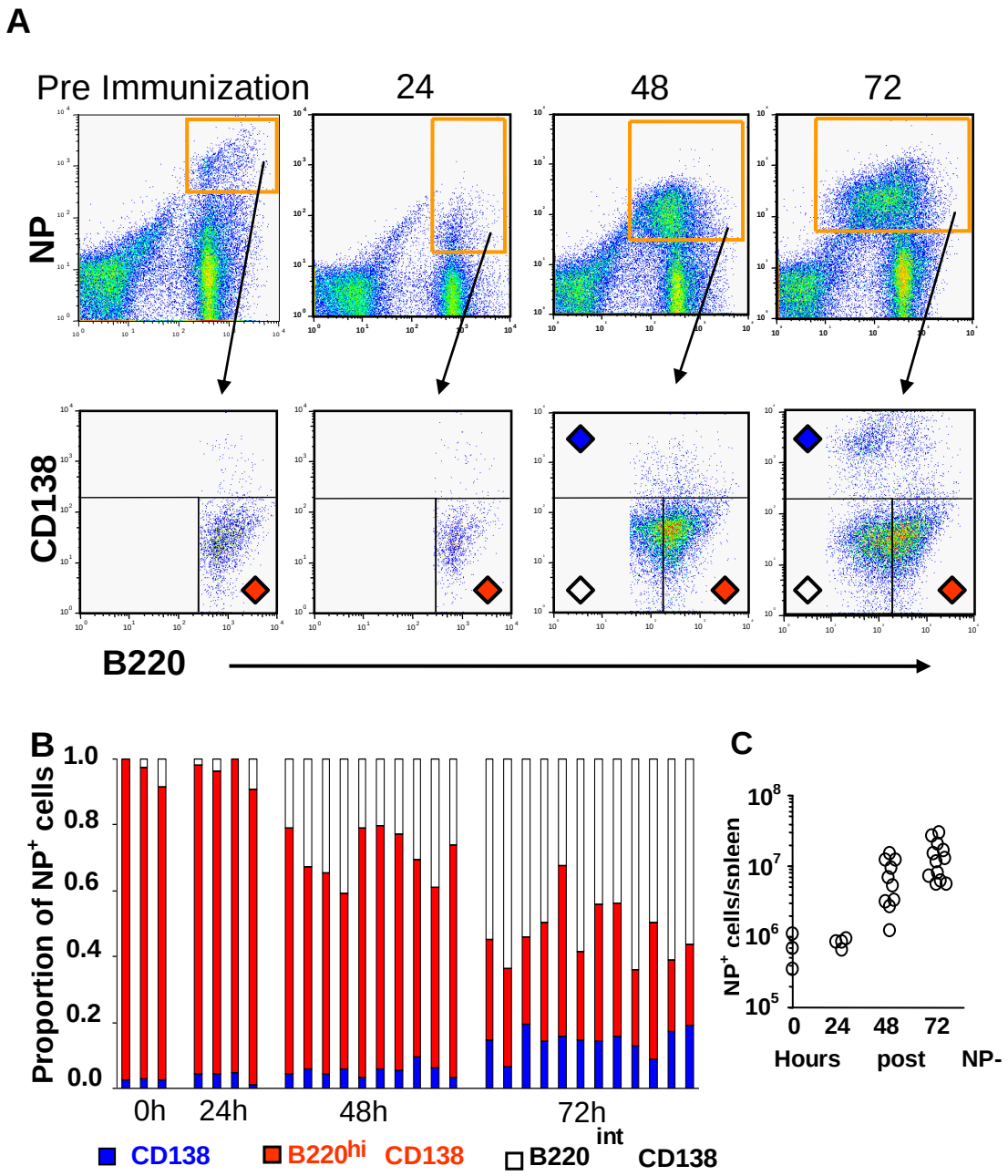


Figure 3.4: FACS analysis of the cells responding to 30μg NP-Ficoll

**A**. Top row shows B220 positive B cells binding NP-PE. The CD138 expression of these populations is shown on the bottom row. Responding B cells were subdivided into $B220^{high}$ (red diamonds), B220 intermediate (white diamonds) and CD138 high (blue diamonds) populations. **B**. The proportion of the responding cells that make up these three populations. **C**. The number of $NP^+$ cells per spleen as the response progresses.

## 3.3. Microarray analysis of mRNA expression within bulk-sorted NP-specific B blasts and plasmablasts identified by flow cytometry

Arrays were designed to assess the mRNA expression of 94 plasmablast and germinal centre-associated genes with 18sRNA and β2microglobulin (β2m) as control genes that are relatively uniformly-expressed. The value of using β2m as this standard for mononuclear leucocytes has recently been published by Serre *et al* in our lab (Serre, *et al*, 2008). These were set out in 384 well microfluidic plates that allow RT2-PCR for each of the selected gene products.

### *3.3.1. NP-binding sorted populations form distinct populations at the mRNA level*

The data from the gene arrays was analysed and sorted according to mRNA expression similarity (Fig. 3.5). This technique plots on a colour scale variation from the average result across all the populations analyzed. Green reflects below average expression, red above average expression while average values appear black. Such analysis does not give absolute levels of expression. It can be seen from the figure that there is clear upregulation of a wide range of genes by 24 hours after immunization. These are shown as green before immunization and red after. There is down regulation of a smaller set of genes reflected by the opposite colour change. At 48 hours both the $B220^{high}$ and $B220^{int}$ populations are relatively homogeneous and their general black colouration on the scale indicates an approximation to the average gene expression levels. At 72 hours all three populations by combinations of down regulation and up-regulation of mRNAs show divergent differentiation from the 48 hours blasts and from each other. To take this analysis further the levels of mRNAs relative that of β2microglobulin were assessed for each of the 94 mRNAs.

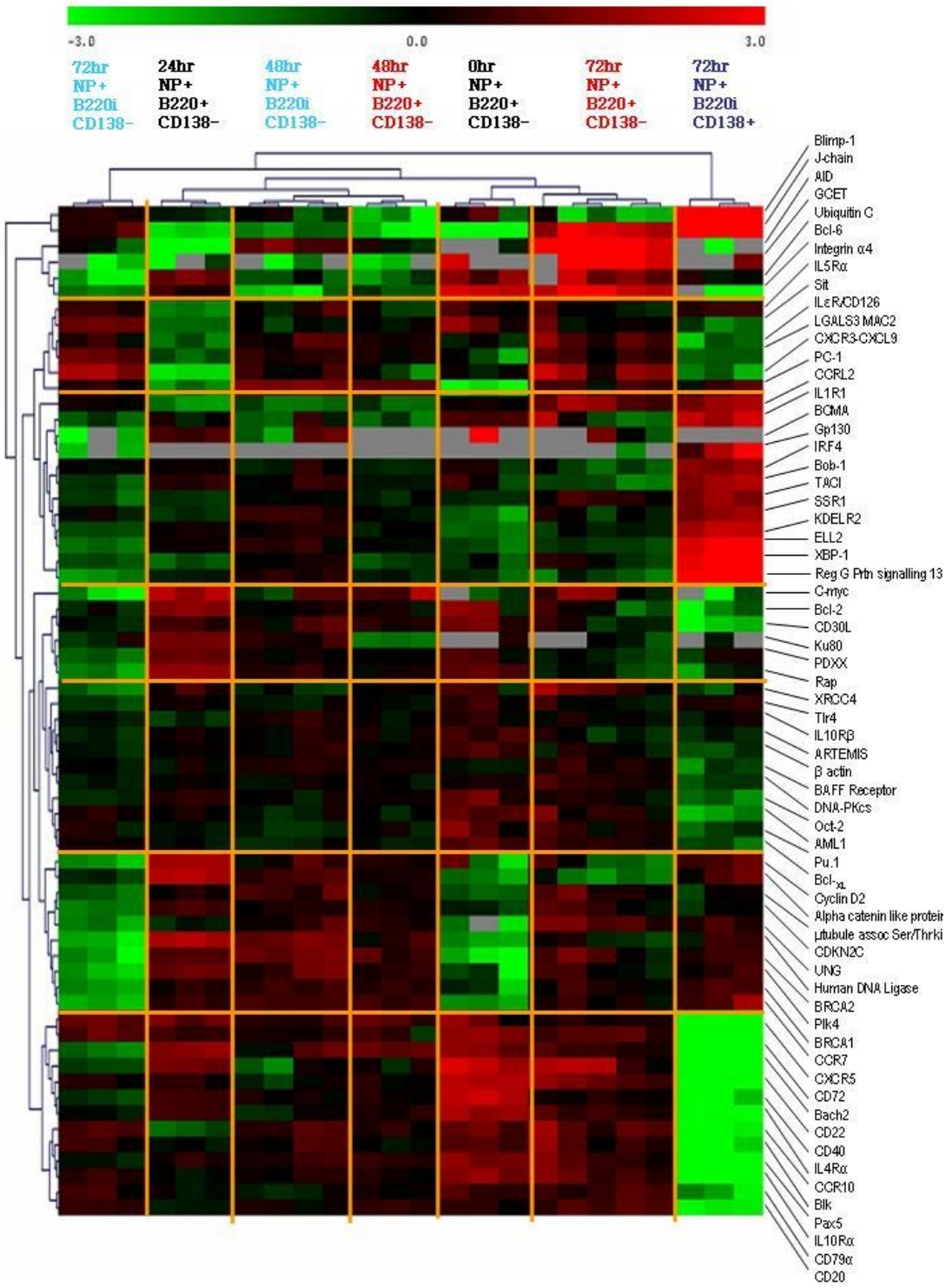


Figure 3.5: Combined gene expression data from low density RT-PCR arrays

Genes selected from the original array data based on significant expression levels. Cell populations are grouped according to similarity in gene expression by the TMEV software. Markers to clarify cell populations and groups of genes are calibrated by eye. Red indicates an upregulation, and green a downregulation (relative to the row average) of gene expression. The range of expression indicated by the colour code ranges from 8 fold ($2^3$) below average (bright green) to 8 fold above average (bright red). Grey indicates no expression detected.

*3.3.2 At 72 hours the B220 intermediate CD138$^+$population expresses mRNA associated with plasmablast differentiation and has down regulated mRNAs for proteins of the BCR complex*

That the CD138$^+$ population at 72 hours expresses plasmablast associated genes confirms that these cells have differentiated to AFC. As would be expected, plasmablast and plasmablast precursor associated genes, such as the transcriptional regulators Blimp1 (Fukuda, *et al*, 1997; Muramatsu, *et al*, 2000; Shapriro-Shelef, *et al,* 2003) and XBP1 (Shaffer, *et al*, 2004), are upregulated in this population. The level of IRF4 (Klein, *et al*, 2006) is also upregulated (Fig 3.6A).

Endoplasmic reticulum-associated genes KDERL2 (Tarte, *et al*, 2003), ELL2 (Nakayama, *et al*, 2006) and SSR1 (Wiedmann, *et al*, 1987) are selectively expressed in this population, reflecting their secretion of antibody (Fig 3.6B). This function is also indicated by J chain upregulation; J chain is essential for IgM and IgA polymerisation (Mestecky, *et al*, 1971; Shaffer, *et al*, 20004). The signal transducer IL6ST, which is downstream of the IL6 receptor, is also involved in the promotion of AFC differentiation (Saito, *et al*, 1993) and is selectively expressed in the CD138$^+$ cells at 72 hours (Fig 3.7A).

The TNF-family receptors BCMA and TACI are both upregulated whilst BAFF receptor is down regulated (Fig 3.7.B). This is in keeping with the published data on the role of these genes in plasmablast differentiation. BAFF receptor is associated with maintaining survival of recirculating and MZ B cells as well as GC B cells (Shulga-Morskaya, *et al*, 2004; Zhang, *et al*, 2005). TACI has been implicated in early plasmablast development (Zhang, *et al*, 2005) in TD and TI responses (von

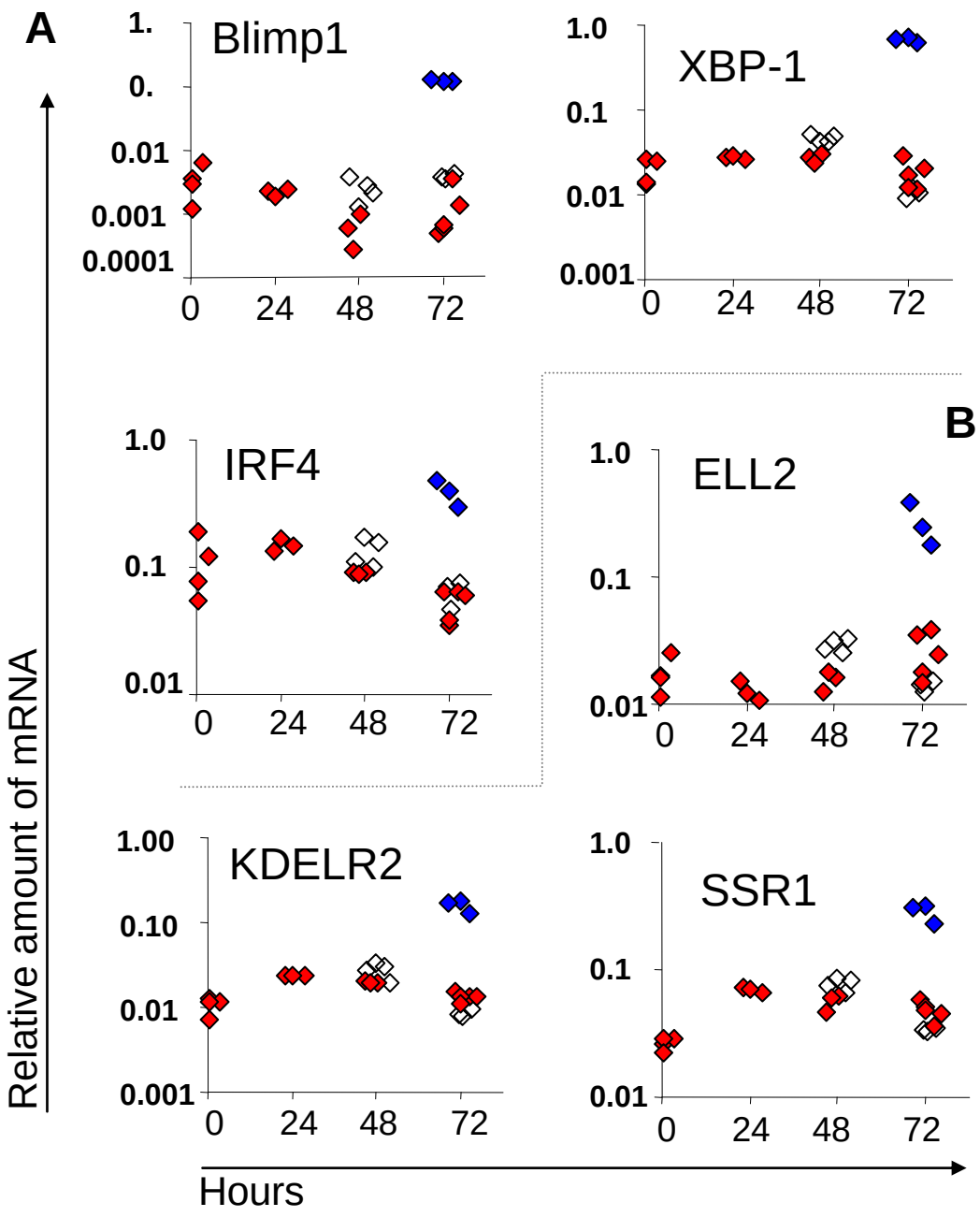


Figure 3.6: Plasmablast associated genes are upregulated in the CD138$^{+}$ population at 72 hours

Genes associated with plasmablast differentiation (**A**) and the production of protein by the endoplasmic reticulum (**B**) are upregulated at the mRNA level at 72 hours in the CD138$^{+}$ population. Each diamond represents one mouse. Red diamonds are B220$^{high}$ CD138$^{-}$ cells, white diamonds, B220$^{int}$ cells and blue diamonds are CD138$^{+}$ B220$^{int}$. All values are relative to $\beta_2$microglobulin.

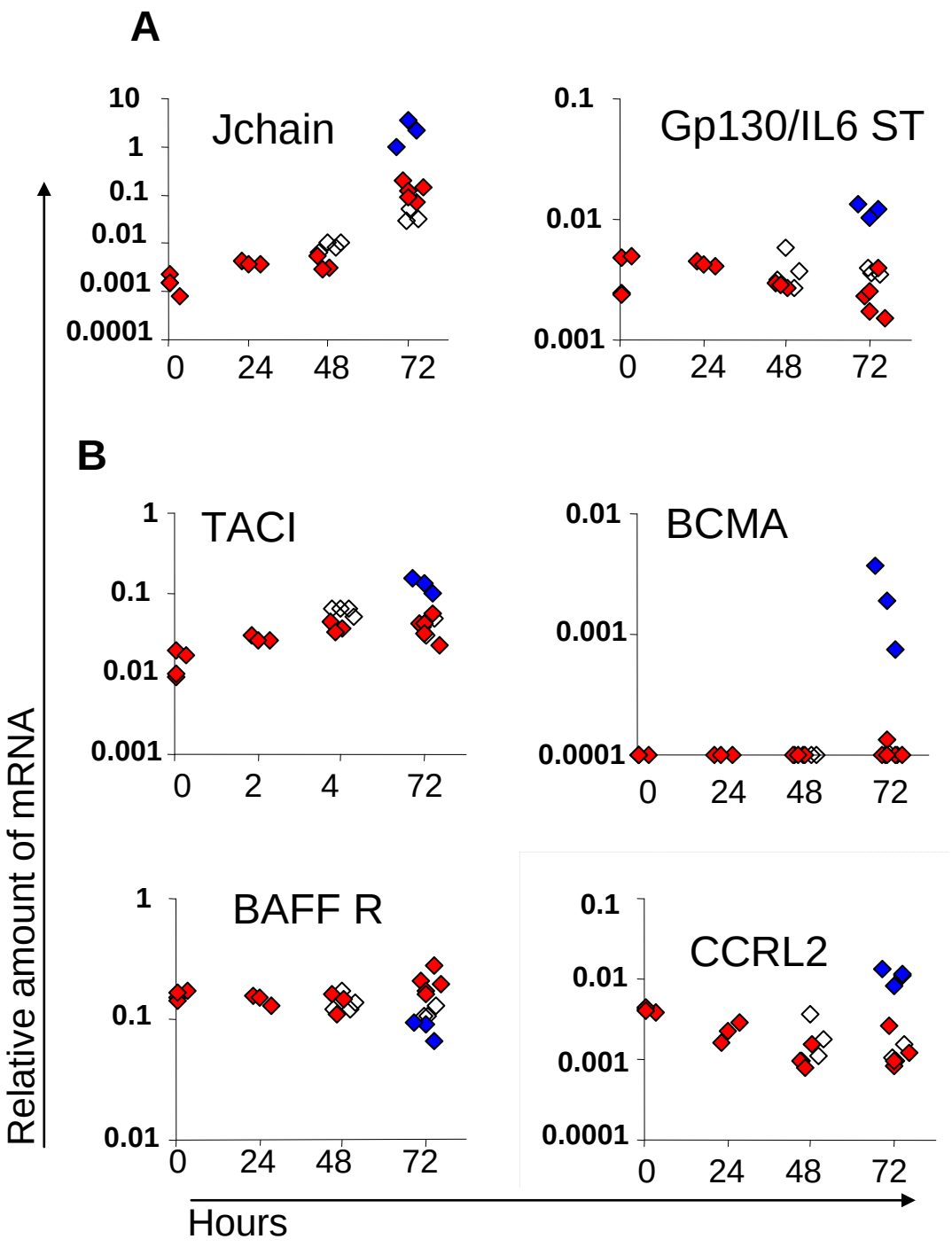


Figure 3.7: Further genes upregulated in the CD138$^{+}$ population at 72 hours

Genes associated with antibody production are upregulated at 72 hours (**A**). TNF family receptors TACI and BCMA are upregulated at 72hours in the plasmablast population whilst BAFF receptor is downregulated (**B**). Each diamond represents one mouse. Red diamonds are B220$^{high}$ CD138$^{-}$ cells, white diamonds, B220$^{int}$ cells and blue diamonds are CD138$^{+}$ B220$^{int}$. All values are relative to $\beta_2$microglobulin.

Bulow, *et al*, 2001). BCMA is reported to maintain plasma cells in the bone marrow (O'Connor, *et al*, 2004). Interestingly CCRL2 is upregulated in plasmablasts (Fig 3.7B, bottom right). This chemokine scavenging receptor is associated with mast cells in the peritoneal cavity and has been reported to augment inflammation (Zabel, *et al*, 2008).

A number of proteins associated with the BCR and signal transduction linked with BCR engagement are downregulated at the mRNA level in the plasmablast population (Fig 3.8). Much of this downregulation relates to the loss of Pax5, a key positive regulator of the BCR complex (Horcher, *et al*, 2001; Nera, *et al*, 2006). CD79$\alpha$ which is essential for the expression of Ig at the cell surface and signal transduction from BCR engagement (Lin and Justement, 1992), is down regulated. A number of proteins reported to regulate calcium fluxes induced by B cell activation are also down regulated; CD20 (Uchida, *et al*, 2004), CD22 (Nitschke, *et al*, 1997) and CD72 (Pan, *et al*, 1999) and Bach2, an inhibitor of Blimp1 (Ochiai, *et al*, 2006; Ochiai, *et al*, 2008),

The receptor for co-stimulation, CD40, which is key in GC formation and T-dependent Ig class switching, (Kawabe, *et al*, 1994) is down-regulated. It would be of interest to see if this down-regulation is more pronounced in TI-2-induced differentiation of B cells to plasmablasts as CD40-ligation has been reported to be unnecessary for TI-2 responses (Xu, *et al*, 1994). IL4R$\alpha$ is also a promoter of CSR in GC (Cunningham, *et al*, 2004). The enzyme essential for CSR - AID (Muramatsu, *et al*, 2000), is strikingly absent from the plasmablasts. This selective down regulation of AID in plasmablasts indicates these cells have lost the capacity to initiate switch recombination. This is considered further in section 3.4.

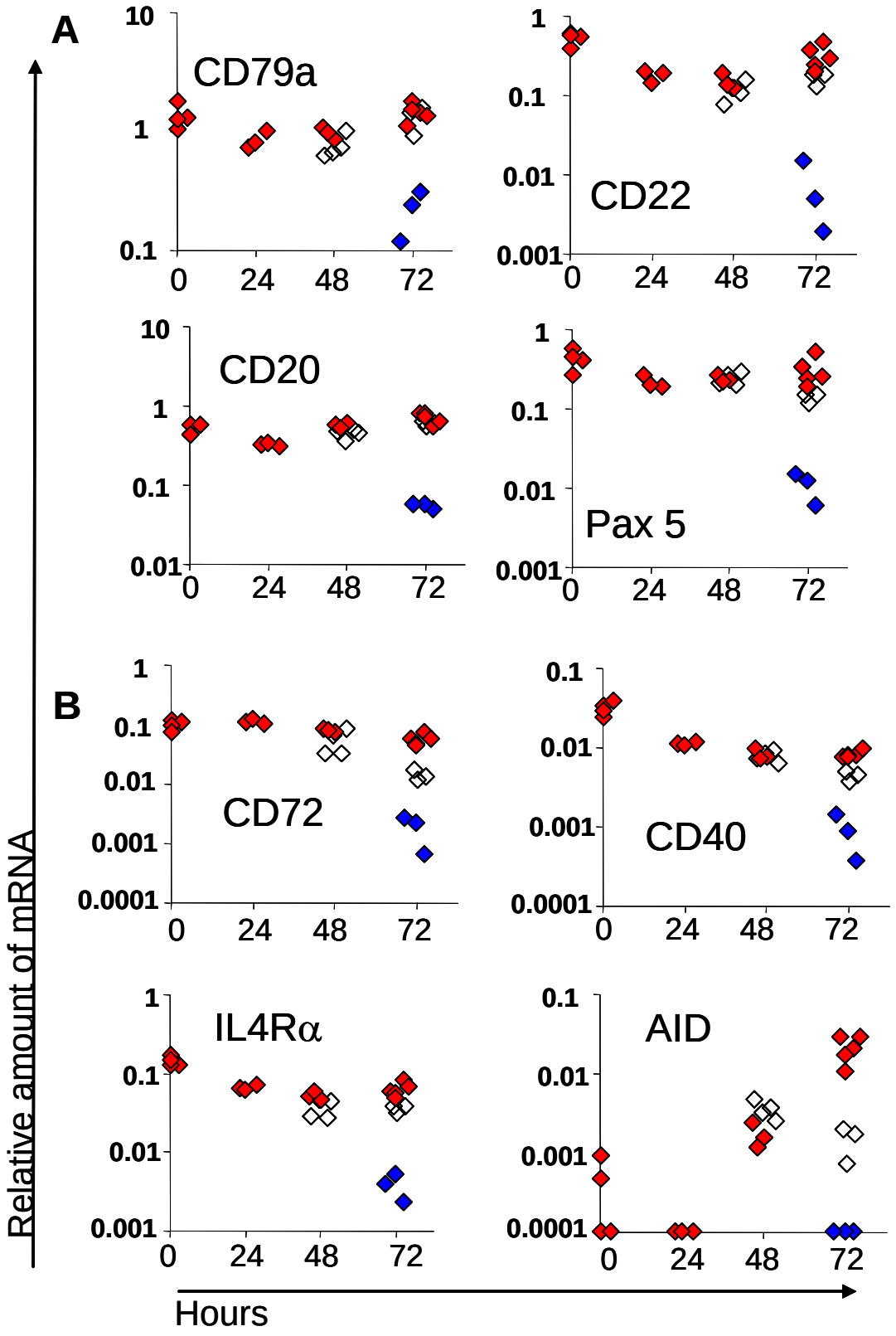


Figure 3.8: Genes associated with the BCR are downregulated at 72 hours in the CD138$^{+}$ population

BCR associated proteins (**A**) and costimulation receptors (**B**) are downregulated in the CD138 population at 72 hours. Each diamond represents one mouse. Red diamonds are B220$^{high}$ CD138$^{-}$ cells, white diamonds, B220$^{int}$ cells and blue diamonds are CD138$^{+}$ B220$^{int}$. All values are relative to β2microglobulin.

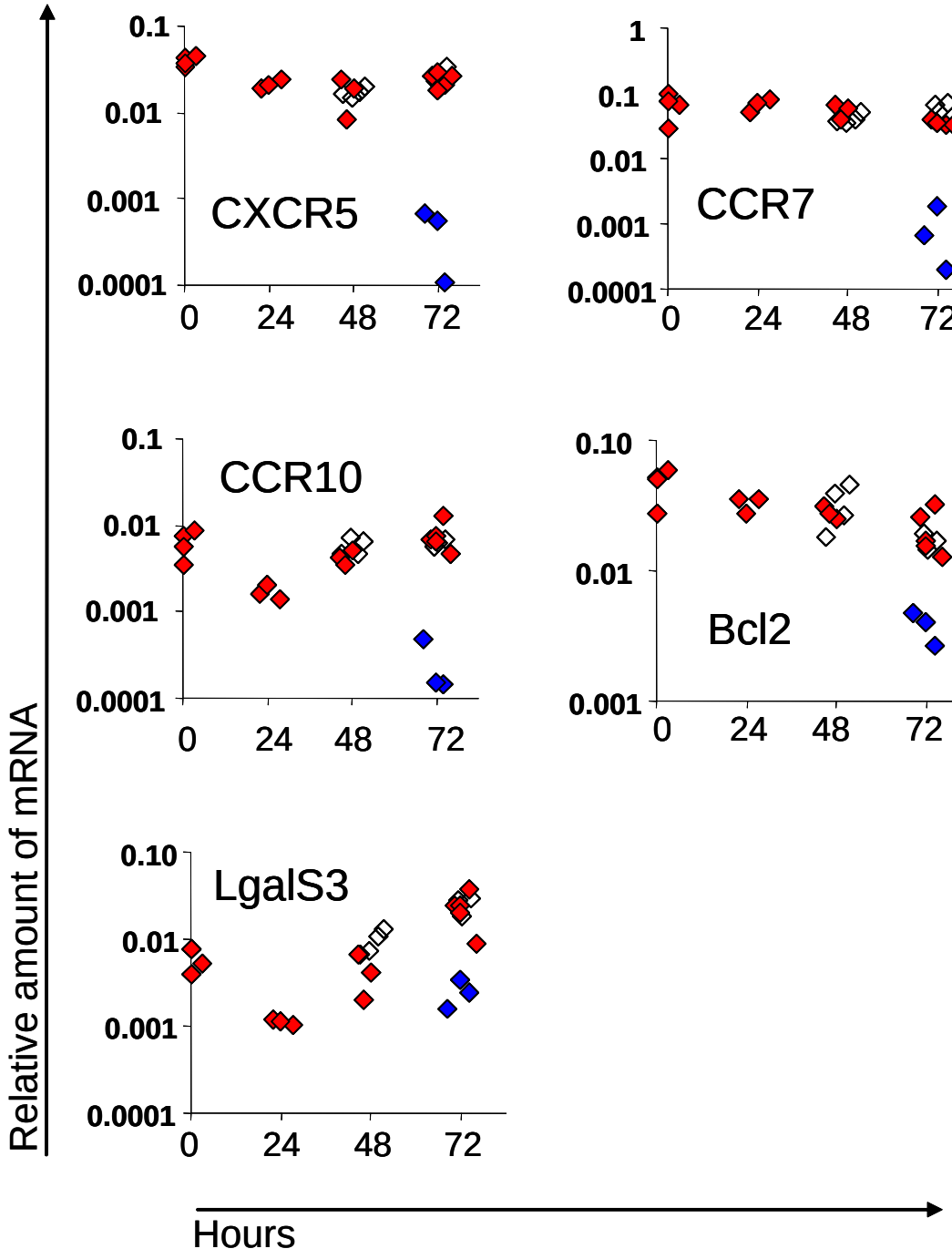


Figure 3.9: Further genes downregulated at 72 hours in the CD138 population

Each diamond represents one mouse. Red diamonds are $B220^{high}$ $CD138^{-}$ cells, white diamonds, $B220^{int}$ cells and blue diamonds are CD138+ $B220^{int}$. All values are relative to $\beta_2$microglobulin.

B blasts migrate from the white pulp to extrafollicular foci as they become plasmablasts during TI-2 responses. Consequently it is not surprising that both CXCR5 and CCR7 are downregulated in the plasmablast population (Fig 3.9).

CXCR5 is involved in the attraction recirculating B cells, MZ cells B cells as well as a subset of primed CD4 T cells into B cell follicles (Cinamon, *et al*, 2008; Ekland, *et al*, 2004). CCR7 is required for the localisation to and retention of B cells at the T zone follicle border in response to antigen (Reif, *et al*, 2002, Okada, *et al*, 2005). CCR10 is a non-signalling chemokine scavenger gene that has been associated with the resolution of inflammation (McKimmie, *et al*, 2008). The data show that it is downregulated in plasmblasts, in contrast to the pro-inflammatory CCRL2 mentioned above.

The reduction in Bcl2 mRNA levels (Fig 3.9) may be related to the high death rate in plasmablasts that do not gain access to stroma in the red pulp that supports their survival (de Vinuesa, *et al* 2000; Sze, *et al* 2000).

Oct-2 activates IL5R$\alpha$ (Emslie, *et al*, 2008) and in this way may promote AFC formation (Hasbold, *et al*, 2003). The down regulation of both of these (Fig 3.10) fits with the loss of CSR capacity and acquisition of plasmablast cell fate. IL-10R is selectively down-regulated in the plasmablasts. IL-10 has been linked to switching to IgA (Fayette, *et al*, 1997). More recently IL10 has been reported in conjunction with the B10 cell subset to repress inflammation (Yanaba, *et al*, 2008; Fillatreau, *et al*, 2002; Tanaka, *et al*, 1996).

Blk, a tyrosine kinase implicated in CD72 signalling (Venkataraman, *et al*, 1998) and LgalS3/MAC2, which is associated with IgE invoked respiratory bursts in neutrophils (Truong, *et al*, 1993; Yamaoka, *et al*, 1995) are also downregulated (Fig

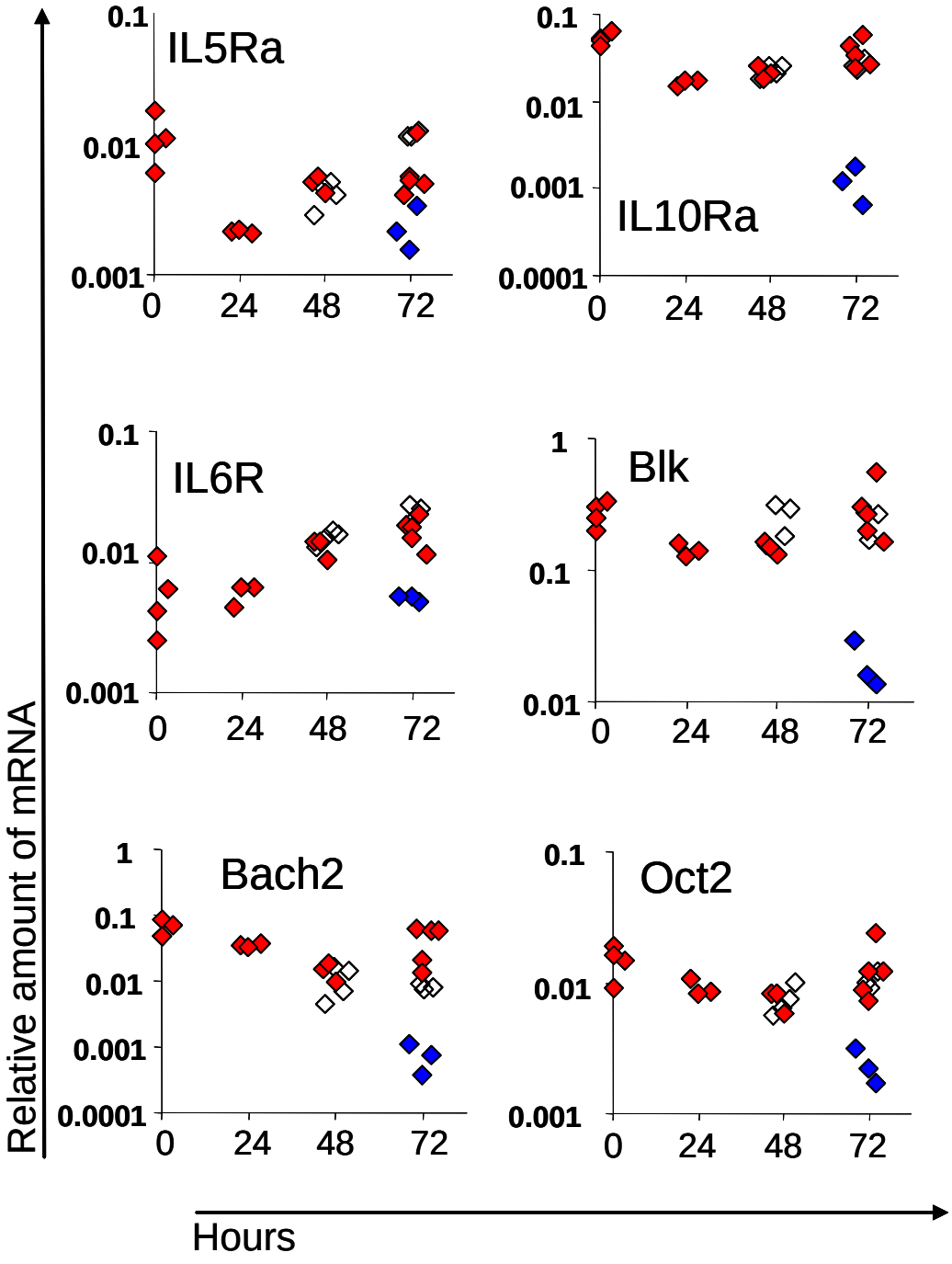


Figure 3.10: Further genes downregulated at 72 hours in the CD138 population

Each diamond represents one mouse. Red diamonds are $B220^{high}$ $CD138^{-}$ cells, white diamonds, $B220^{int}$ cells and blue diamonds are $CD138^{+}$ $B220^{int}$. All values are relative to $\beta_2$microglobulin.

3.9 and 3.10). IL6 has been reported to have roles both in human GC (Burdin, *et al*, 1996), and promoting the differentiation to AFC (Hirano, *et al*, 1985). IL6 ST (gp130) is selectively up regulated in the $CD138^{+}$ population while levels of IL6R are lowered.

### *3.3.3. mRNA from cells in the B220 $^{high}$ CD138- B blast population includes mRNAs associated with germinal centre B cells*

The population of B blasts at 72 hours that remain $B220^{high}$ starts to express GC associated genes (Fig 3.11). This $B220^{high}$ population expresses Bcl6 (Cattoretti, *et al*, 1995), AID (Muramatsu, *et al*, 1999), Fas (Smith, 1995; Hao, *et al*, 2008) and the germinal centre expressed transcript (GCET) (Christoph, *et al*, 1994). Also upregulated in the $B220^{high}$ population is the BAFF receptor which has been reported to be necessary for the maintenance of GC responses (Shulga-Morskaya, *et al*, 2004). RAPGEF, a guanine nucleotide exchange factor, is also upregulated.

Germinal centre B cells are selected on the basis of antigen binding, processing and presentation to CD4 T cells (Liu, *et al*, 1989; Gulbranson-Judge and Maclennan, 1996). Consequently it is not surprising that there is continued expression of the BCR complex regulator Pax-5 (Lin, *et al*, 2002) and CD40 in the $B220^{high}$ population at 72 hours (Fig 3.8).

### *3.3.4. The CD138$^{-}$ B220 intermediate cells at 72 hours have downregulated GC-associated genes including DNA repair genes*

In contrast to the $CD138^{+}$ and the $CD138^{-}$ $B220^{high}$ populations, which express plasmablast and GC mRNAs respectively, the B220 intermediate population shows little evidence of commitment to become GC B cells or plasmablasts. Germinal centre associated genes such as AID, Bcl6 and FAS are downregulated in

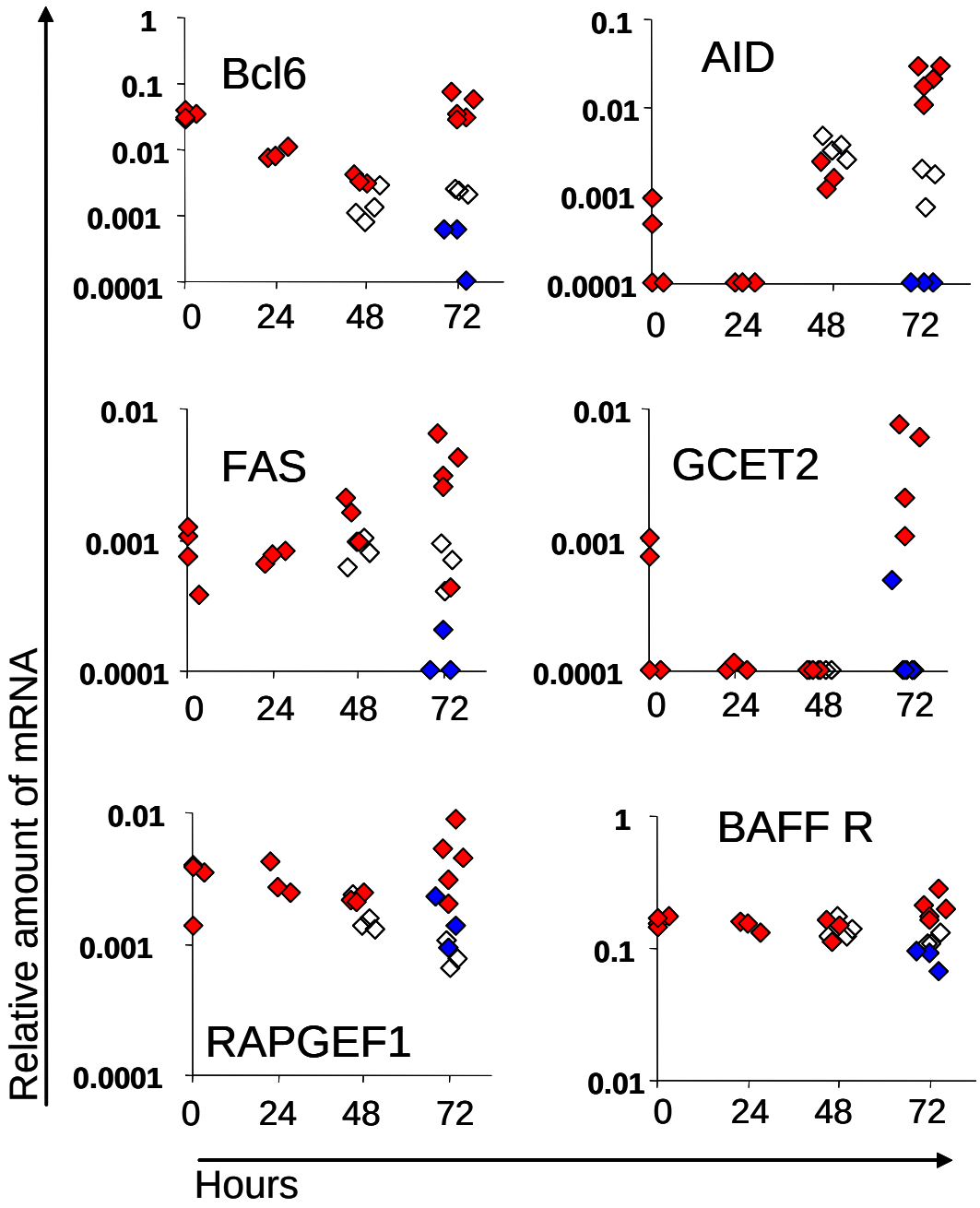


Figure 3.11: Genes upregulated in the B220$^{high}$ population at 72 hours are associated with germinal centres

Each diamond represents one mouse. Red diamonds are B220$^{high}$ CD138$^{-}$ cells, white diamonds, B220$^{int}$ cells and blue diamonds are CD138$^{+}$ B220$^{int}$. All values are relative to $\beta_2$microglobulin.

these cells (Fig 3.12). In addition the expression of DNA repair genes such as breast cancer 1(BRCA1) (Scully, 2001), breast cancer 2 (BRCA2) (Moynahan, *et al*, 2001) and uracil DNA glycosylase (UNG) (Rada, *et al*, 2002) decreases which may suggest that these cells are unlikely to be involved in active DNA hypermutation (Fig 3.12 and 3.13). DNA ligase I, which is also downregulated, may also be involved in this process; DNA ligase IV, which was not included in our array, is involved in double stranded break repair (Pan-Hammarstrom, *et al*, 2005).

Two genes linked with cell cycle control show low levels of expression: (i) Polo like kinase (plk) is a protein kinase that localises to the mitotic and meiotic apparatus during cell division (Lee, *et al*, 1995); (ii). CDKN2C, *in vitro* this has been shown to be upregulated by IL6 during plasma cell differentiation and is involved in bringing plasmablasts out of cell cycle (Morse, *et al*, 1997).

In addition, microtubule-associated serine/threonine kinase (MAST2) and Bob 1 expression declines (Fig 3.13). MAST2 is upregulated by Fc$\gamma$R ligation in macrophages and leads to their production of IL12 (Zhou, *et al*, 2004). Bob1 is B cell specific and binds Oct-2 to promote antibody production but not CSR or proliferation (Qin, *et al*, 1998; Gstaiger, *et al*, 1995).

The response to NP has been shown to produce a $NP^{+}B220^{neg}$ memory population within 7 days of the wild type response to NP-keyhole limpet heamocyanin (Driver, *et al*, 2001). By this time it comprises 50% of the responding cells and does not express GC or plasmablast markers. Work from our laboratory showed that the response to NP-Ficoll could generate memory from the B1 B cell subset (Hsu, *et al*, 2006). Similarly, memory B cells have been shown to express low

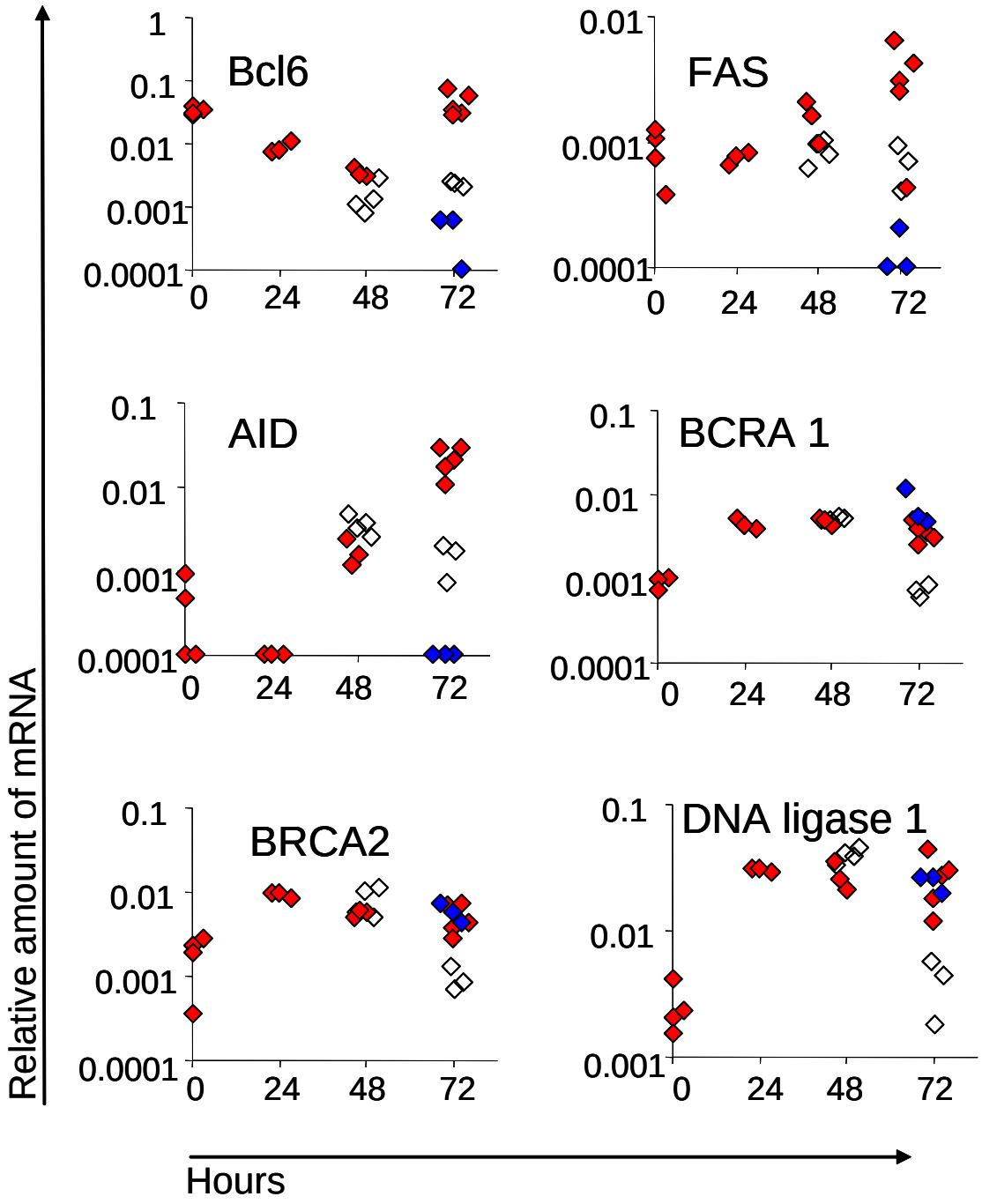


Figure 3.12: Genes downregulated in the B220 intermediate population at 72 hours are associated with germinal centres and DNA repair

Each diamond represents one mouse. Red diamonds are $B220^{high}$ $CD138^{-}$ cells, white diamonds, $B220^{int}$ cells and blue diamonds are $CD138^{+}$ $B220^{int}$. All values are relative to $\beta_2$microglobulin.

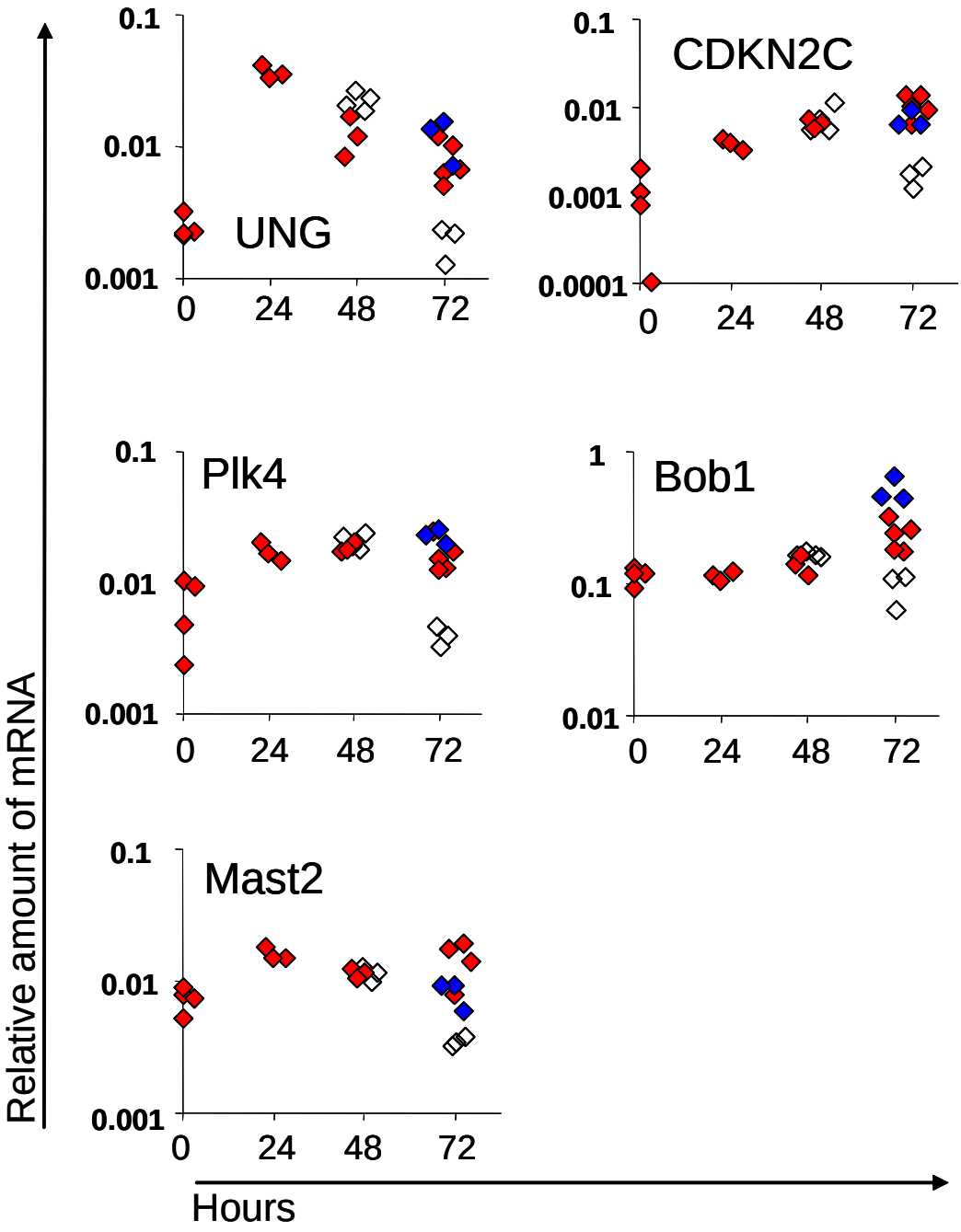


Figure 3.13: Genes downregulated in the B220 intermediate population at 72 hours are associated with germinal centres and DNA repair

Each diamond represents one mouse. Red diamonds are $B220^{high}$ $CD138^{-}$ cells, white diamonds, $B220^{int}$ cells and blue diamonds are $CD138^{+}$ $B220^{int}$. All values are relative to $\beta_2$microglobulin.

Bcl6 and low CDKN2C (Kuo, *et al*, 2007). The possibility that these cells may represent a memory population provides an avenue for further investigation.

*3.3.5 Within 24 hours genes associated with CSR and DNA repair are upregulated*

As previously described, the response to NP-Ficoll provokes considerable changes in mRNA expression within 24 hours of immunization (Fig 3.14). Some mRNAs of proteins associated with DNA repair such as BRCA1, DNA ligase I and UNG (section 3.3.4) are upregulated – perhaps in preparation for class switch recombination (Fig 3.14A). J chain, which is necessary for IgM polymerization and g protein signalling 13, which is the IL6 signal transducer without which antibody responses are impaired (Kumanogoh, *et al*, 1997), are both upregulated at this time (Fig 3.14B). CyclinD2, a protein that allows progression from $G_1$ and is upregulated by BCR signalling (Glassford, *et al*, 2003), also shows an increase in mRNA expression (Fig. 3.15). Finally, CD30L, that is expressed by T cells and B cells (Shanebeck, *et al*, 1995; Younes, *et al*, 1996) and promotes proliferation and antibody secretion, expression also increases.

Given that the responding cells move from the MZ to the T zone during the first day of the response (section 3.1) a change in chemokine receptor signalling would be expected. The chemokine receptor CXCR4 is implicated both in migration to the T zone (Casamayor-Palleja, *et al*, 2002) and from the T zone to extrafollicular foci (Hargreaves, *et al,* 2001) and so an upregulation of this gene might be expected. Unfortunately an assay for this gene was not available for the array at the time of design. Twenty-four hours after NP-Ficoll administration C-C chemokine receptor 1 (CCR1) and CCR8 are both downregulated in responding cells (Fig 3.15). C-C chemokine receptor 1 is expressed by naïve and memory B cells (Corcione, *et al*,

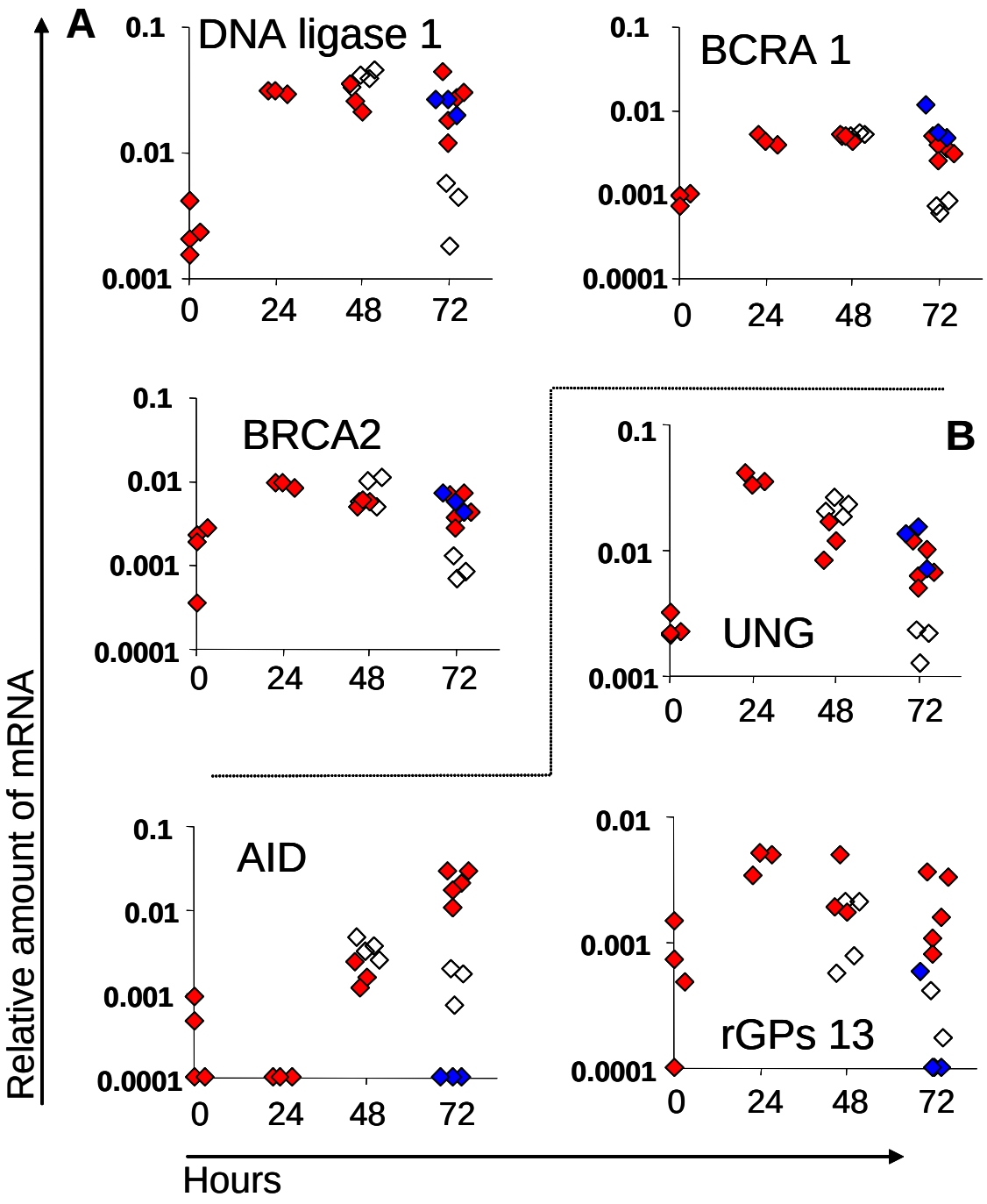


Figure 3.14: Genes upregulated in the 24 hours of the response are associated with DNA repair, CSR, cell cycle and cell movement

Genes associated with DNA repair (**A**), class switch recombination and somatic hyper mutation (**B**), antibody production and cell cycle (**C**) and cell movement (**D**) undergo changes in their mRNA expression during the first day. Each diamond represents one mouse. Red diamonds are $B220^{high}$ $CD138^{-}$ cells, white diamonds, $B220^{int}$ cells and blue diamonds are $CD138^{+}$ $B220^{int}$. All values are relative to $\beta_2$microglobulin.

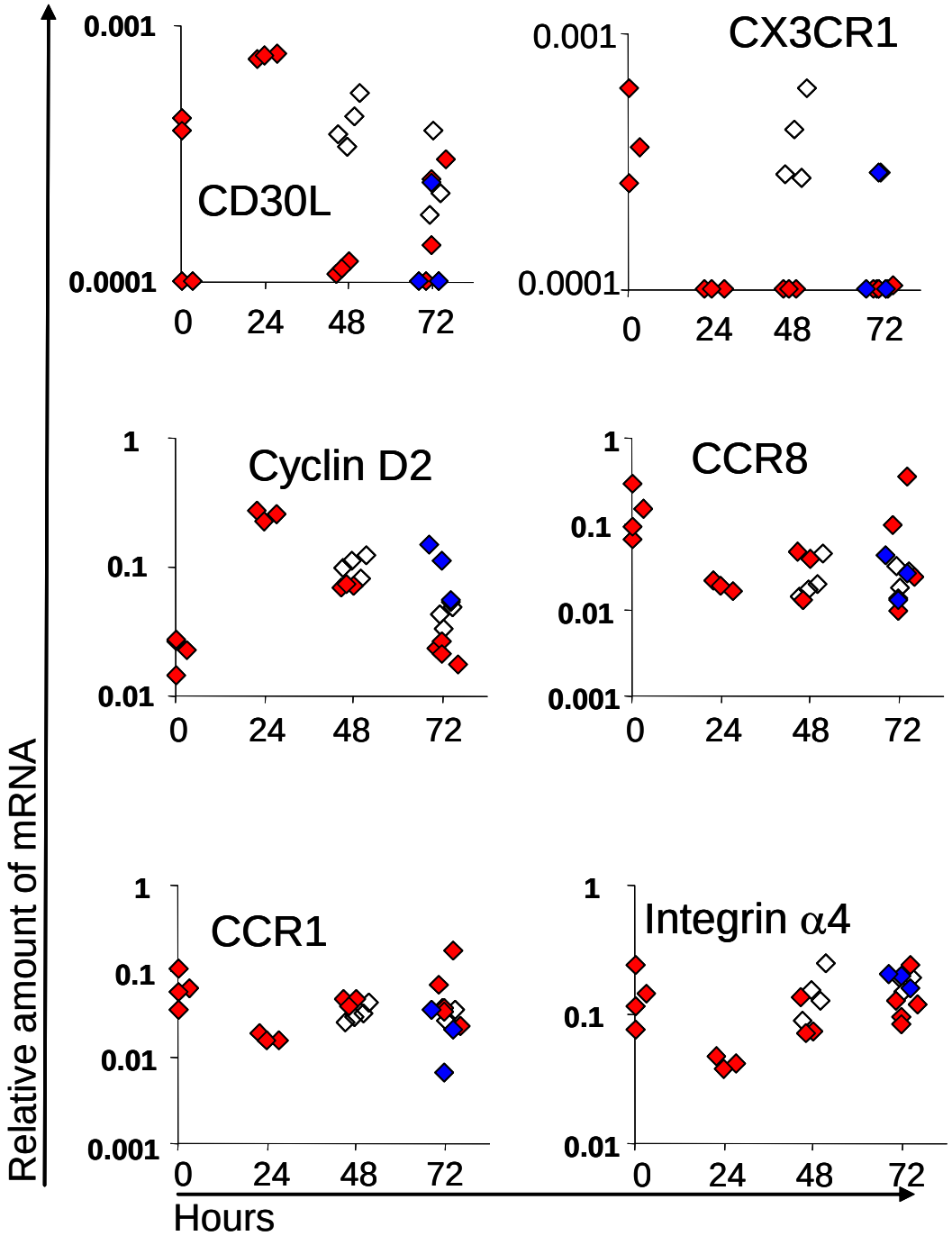


Figure 3.15: Additional genes that show mRNA level changes during the first 24 hours of the response

Each diamond represents one mouse. Red diamonds are $B220^{high}$ $CD138^{-}$ cells, white diamonds, $B220^{int}$ cells and blue diamonds are $CD138^{+}$ $B220^{int}$. All values are relative to $\beta_2$microglobulin.

2002). Integrin alpha 4 allows B cells to bind to stroma (Behr and Schriever, 1995) and the downregulation of this molecule at 24 hours may reflect the movement of the cells from the MZ. In addition the Chemokine (C-X3-C motif) receptor 1 is downregulated at 24 hours. CX3CR1 has been reported in some B cell lymphomas and is an adhesion molecule that regulates cell homing (Andreasson, *et al*, 2008).

### *3.3.6 At 48 hours the B220 intermediate population is distinguished by the expression of CD30L and CX3CR1*

The slight increase in expression of CD30L, ELL2 and CX3CR1 in the B220 intermediate cells compared to the $B220^{high}$ cells shows the limitations of the gene array, as opposed to a genome wide investigation, when investigating differentiation (Fig 3.16). These three genes appear to provide a possible point of differentiation between the two populations at this time. Expression of CX3CR1, a gene involved in tissue homing, and CD30L, involved in costimulation (section 3.3.5) may hint at interactions with other cells in the T zone during the second day. The lack of difference between the two populations might also suggest that the decline in B220 expression is a false indicator (this is discussed in Section 3.6.4).

### *3.3.7. The limitations of gene expression profile analysis*

These gene expression studies were performed on bulk sorts of the identified populations. The quantity of mRNA expressed was calculated relative to the expression of the control gene $\beta_2$microglobulin. Because cDNA has been prepared from a large number of cells the result only informs about the total mRNA in all the cells. It cannot distinguish a small proportion of the cells expressing a high level of an mRNA and the remainder none, from a situation in which all the cells express a

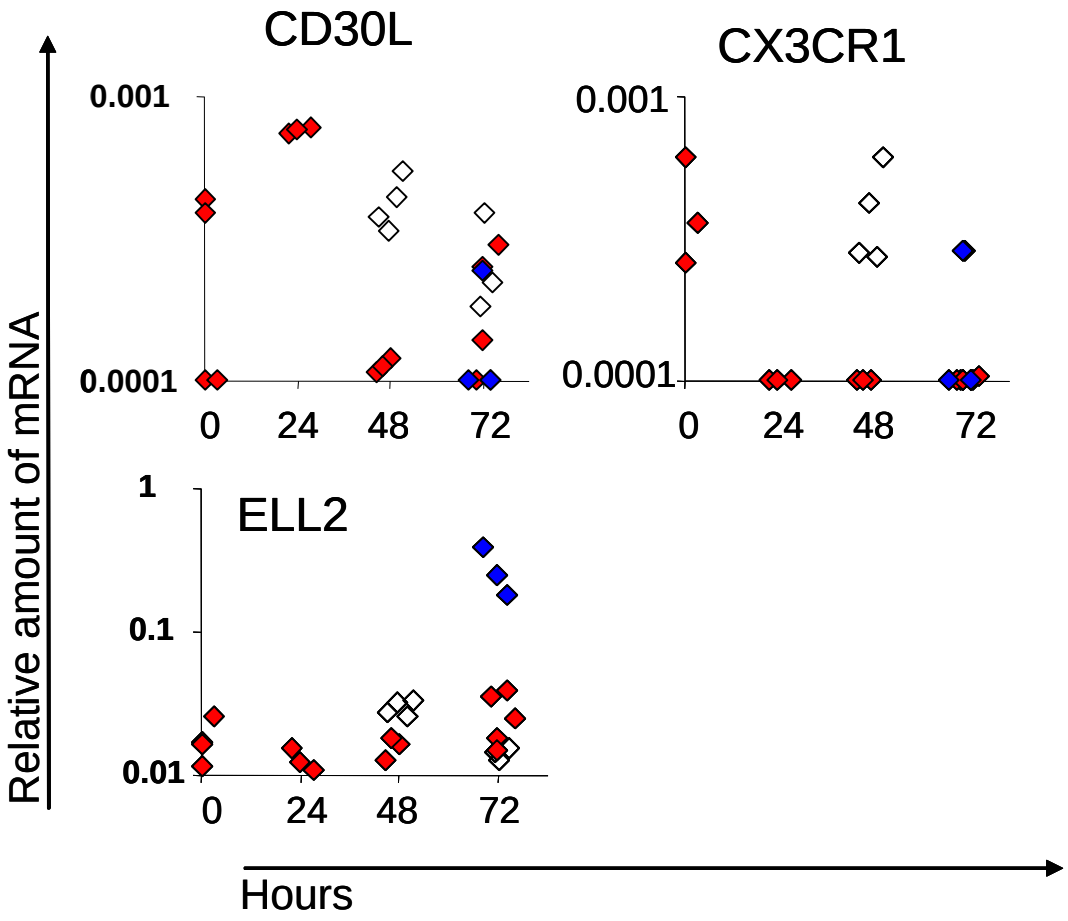


Figure 3.16: Genes that show significant mRNA level changes during the second day of the response

CD30L, CX3CR1 and ELL2 are expressed at 48 hours at a higher level in the B220 intermediate population than in the B220 high population. Each diamond represents one mouse. Red diamonds are $B220^{high}$ $CD138^{-}$ cells, white diamonds, $B220^{int}$ cells and blue diamonds are $CD138^{+}$ $B220^{int}$. All values are relative to $\beta_2$microglobulin.

small amount of the mRNA. In addition the logarithmic nature of PCR amplification biases greatly toward high expressing cells. Consequently whilst this bulk sort analysis provides clues about gene expression changes associated with differentiation of B cells it cannot be considered to be an accurate reflection of single cells participating in the response and may mask important gene expression changes. For example, both Blimp1 and Bcl6 mRNA are expressed at 72 hours in the $CD138^{-}$ $B220^{high}$ population. It is conceivable that within this population some cells will express one mRNA whilst others do not. Bulk sorts mean that such subtleties are lost. Without the definition of single cell RT2-PCR, or genomic arrays based upon single cell amplification, it is not possible to be accurate in our conclusions as to the differentiation of the responding B cells in the populations we identified.

### 3.4. The use of single cell analysis to define the cells that undergo class switching in the extrafollicular response and where and when B Blasts differentiate either to germinal centre cells or plasmablasts.

Single cell RT2-PCR was utilised to study the differentiation of cells within the populations previously described. The expression of four key genes was analysed:

1. AID- expression is necessary for class switch recombination to occur

2. Bcl6- the transcription factor responsible for GC cell fate

3. Blimp1- a key transcription factor responsible for plasmablast cell fate

4. IRF4 - a transcription factor necessary for the production of AFC and class switch recombination. The expression of this key protein will be described in a section 3.5.

*3.4.1 Class switching in the extrafollicular response occurs in the T zone B blasts before they differentiate into AFC.*

First we looked to identify if and when switched cells appear during the response to NP-Ficoll. The first switched NP specific cells identified in the spleen by immunohistology were plasmablasts in extrafollicular foci 72 hours after NP-Ficoll (Fig 3.17A).

A search using RT2-PCR on bulk sorted responding cells showed that mRNA from the $CD138^{-}$ $B220^{high}$ as well as the $B220^{int}$ populations at 48 hours had processed transcripts of Iμ that had recombined to Cγ3 (Fig 3.17B). The levels of these transcripts had risen in both these populations by 72 hours, when higher levels were found in the $CD138^{+}$ cells. These data indicate that some switching is occurring in B blasts, but do not exclude continued switching in plasmablasts. Class switch recombination is dependant upon expression of AID (Muramatsu, *et al,* 2000; section 1.5.6) and the bulk analysis showed AID mRNA was undetectable in $CD138^{+}$ cells (Fig 3.8). This is confirmed in the single cell analysis. AID was very rarely found in $CD138^{+}$ cells (3 out of 386 cells, Fig 3.18B, middle graph). On the other hand at both 48 and 72 hours AID mRNA is expressed in ~10% of the $CD138^{-}$ $B220^{high}$ and $B220^{int}$ cells. These data show that switching in the extrafollicular response can and does occur in B blasts prior to the expression of CD138 protein. In addition, the absence of AID in $CD138^{+}$ cells precludes these cells initiating CSR. Further characterization of the $CD138^{+}$ cells and the emergence of the $Bcl6^{+}$ $AID^{+}$ cells in the $B220^{high}$ fraction of B cells at 72 hours (Fig 3.18A) is considered in the next section.

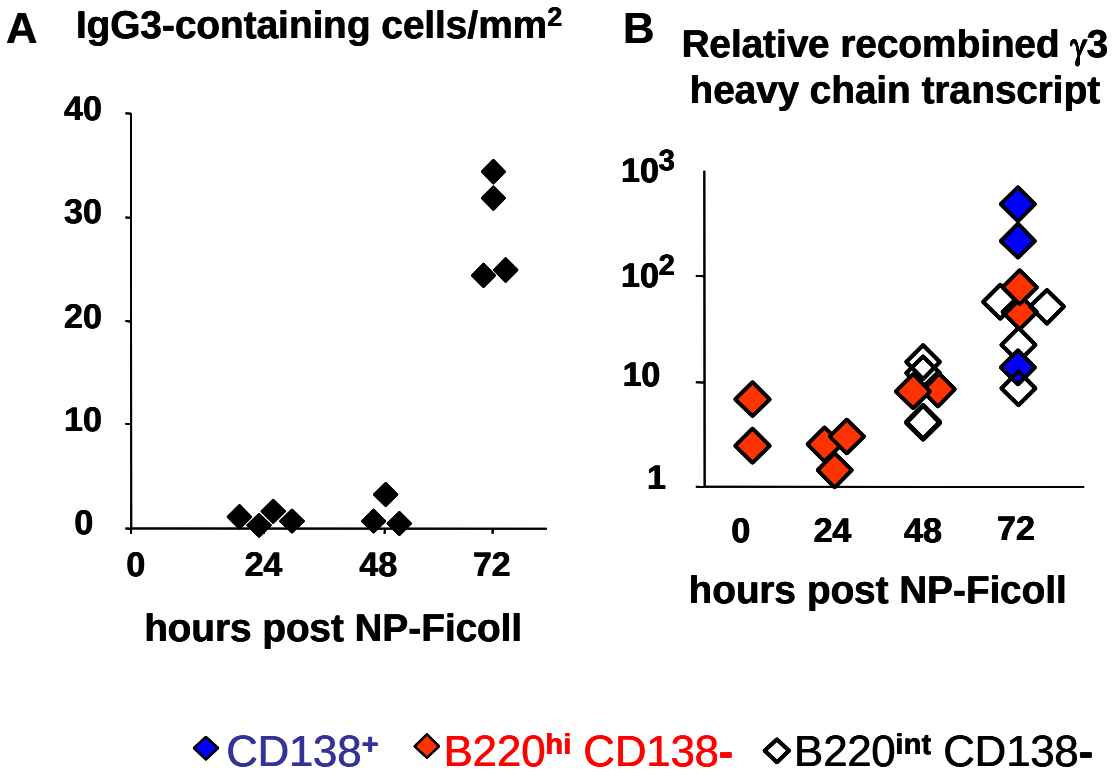


Figure 3.17: Switch recombination to IgG3 in the response to 30μg NP-Ficoll

**A.** IgG3 positive NP binding cells per $mm^2$ in the spleen. **B.** Expression of recombined switched transcripts to γ3 in the sorted $NP^+$ populations. Class switch recombination occurs at 48 hours with protein expression at 72 hours after immunization.

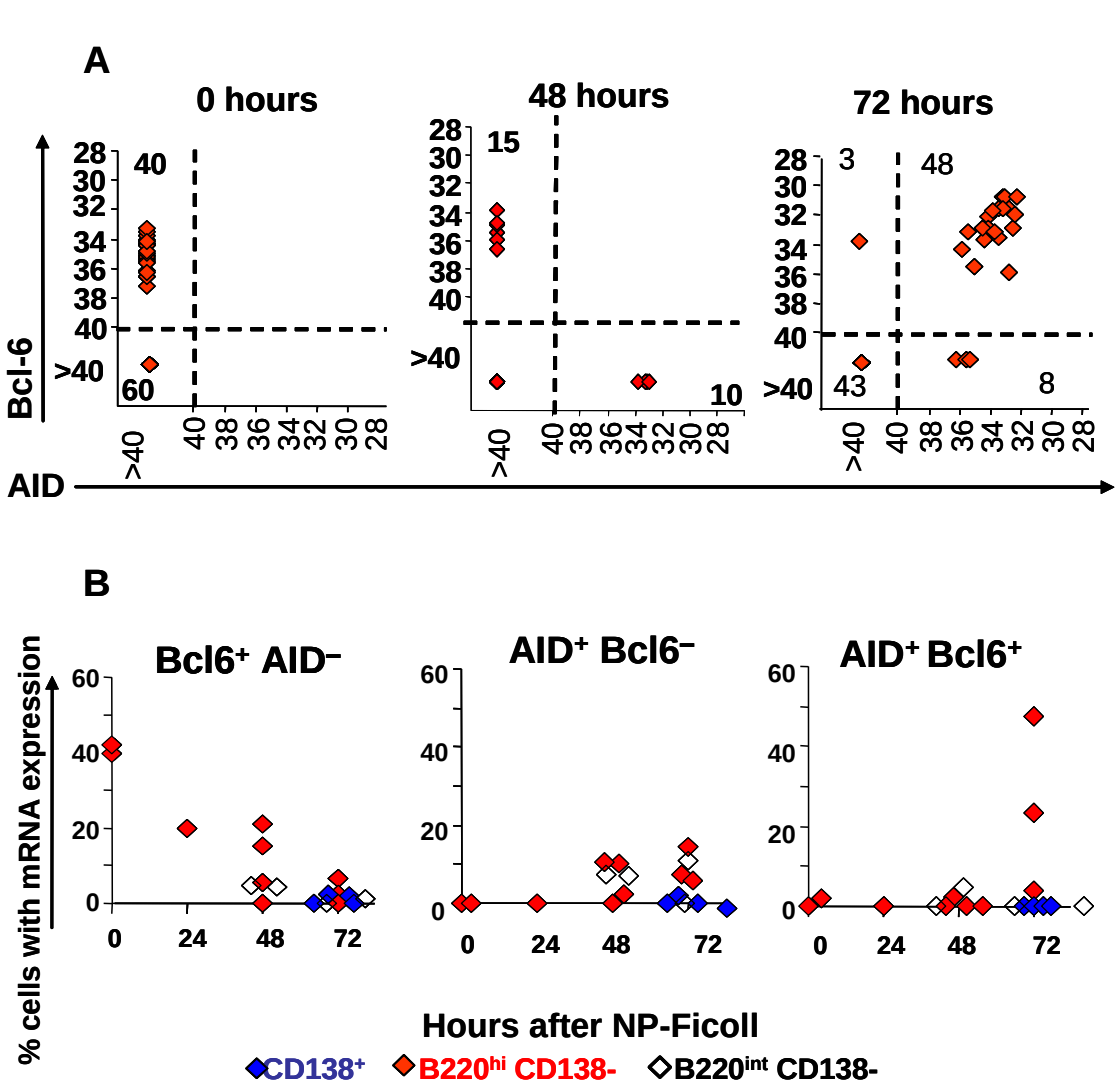


Figure 3.18: Expression of AID and Bcl6 at the single cell level during the response to NP-Ficoll

**A.** Example plots of AID and Bcl6 multiplex RT2-PCR in $NP^+$ $B220^{high}$ cells. Each diamond represents one β2 microglobulin positive cell. Numbers show the percentages of cells in each quadrant. **B.** The percentage of Bcl6+ AID-, AID+ Bcl6- and AID+Bcl6+ cells in each population after immunization. B220 high cells are red diamonds, B220 intermediate cells white diamonds and $CD138^+$ cells are blue diamonds. Each diamond represents one mouse.

*3.4.2. Characteristics of the plasmablast cells induced by 30μg NP-Ficoll.*

As described, a small $CD138^+$ B220 intermediate population is first apparent at 48 hours and is prominent by 72 hours. In addition these cells are $IRF4^{high}$ (Fig. 3.19A and section 3.5).

95% of $CD138^+$ cells express Blimp1 consistent with published data on AFC (Fig, 3.19B, Sanderson, *et al,* 1989). 5% of cells in the $CD138^+$ fraction are $Blimp1^-$. This is consistent with the possibilities that CD138 expression precedes expression of Blimp1 or that a small subset of $CD138^+$ cells exist that do not require Blimp1. Almost no $CD138^+$ B220 intermediate cells express AID or Bcl6 (fig 3.18B).

*3.4.3 Germinal centre precursors first appear in the B220 high $CD138^-$ subset at 72 hours*

The $B220^{high}$ $CD138^-$ population at 72 hours includes 10-60% $Bcl6^+$ $AID^+$ cells (fig 3.18B). This phenotype is characteristic of GC (Muramatsu, *et al*, 1999). It is unclear whether the remaining cells $CD138^-$, $B220^{high}$ cells are destined become either GC cells or plasmablasts. As mentioned above some express AID and a few express Bcl6 without AID.

The cells of the $CD138^-$ $B220^{int}$ population do not express Blimp1 nor do they have a GC phenotype ($Bcl6^+AID^+$). However, a small proportion of these cells express AID (Fig 3.18B). These experiments do not clarify the role of the B220 intermediate subset. That they might differentiate into plasmablasts or GC cells, or that they might be a separate population of B1b cells apparent at 48 hours, will be discussed.

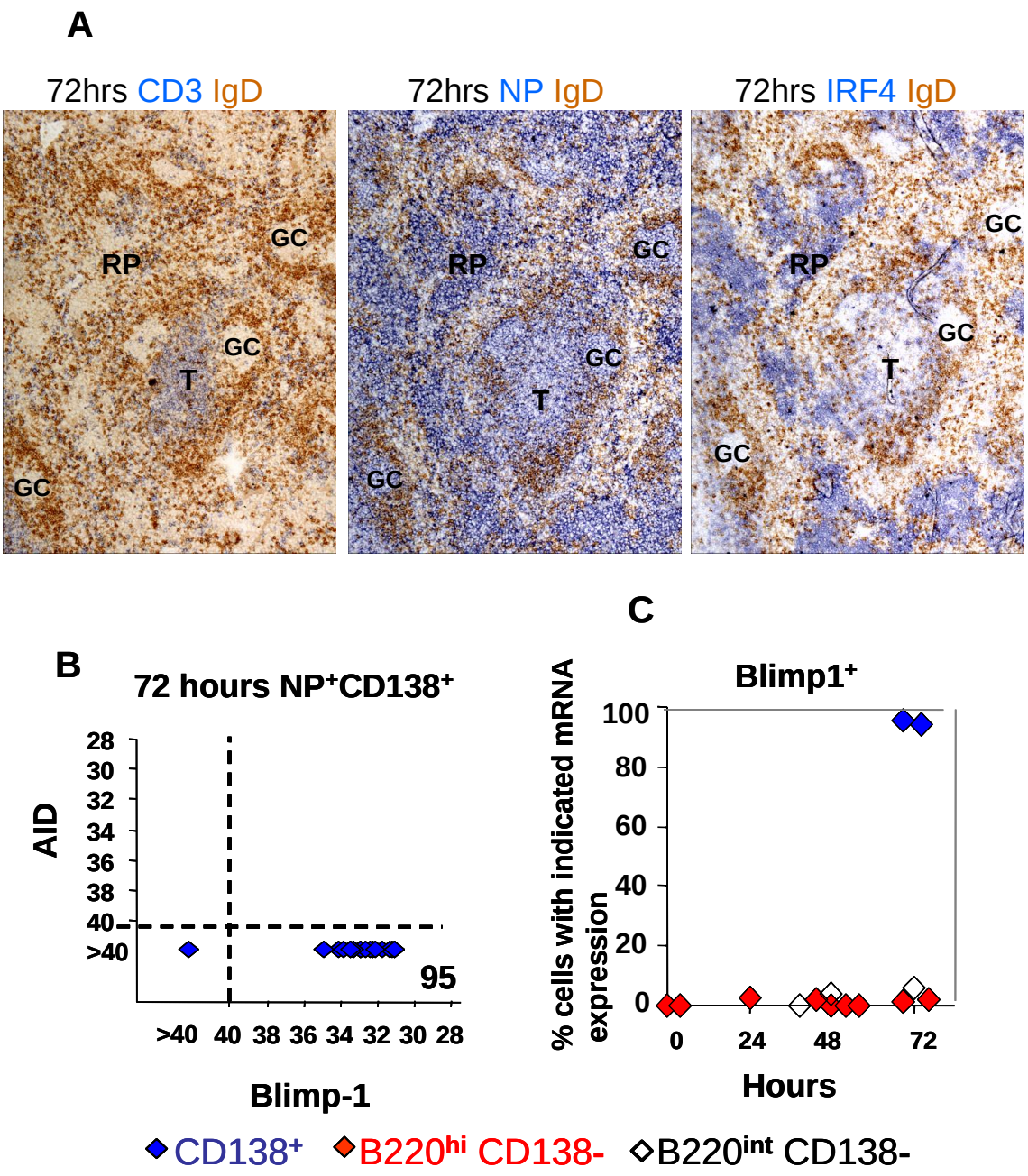


Figure 3.19: Plasmablast populations in the QMxB6 response to 30μg NP-Ficoll

**A**. Expression of IRF4 at 72 hours. Germinal centres (GC) appear largely IRF4 negative whilst red pulp plasmablasts are IRF4 high. IgD is brown, CD3, NP and IRF4 in blue. F= Follicle, T = T zone/ PALS, GC = germinal centre, RP = red pulp. **B.** An example plot of Blimp1 and AID RT2-PCR on CD138 positive cells at 72 hours. **C.** Blimp1 expression in all the sorted populations. Blimp1 is only expressed at 72 hours in the CD138 response.

*3.4.4. The significance of Bcl6 mRNA expression in naïve B cells in the absence of Bcl6 protein.*

Prior to immunization ~60% of NP-binding cells express Bcl6 mRNA. This has declined to ~20% by 24 hours and at 48 hours is in 10-20% of the responding B cell subsets (Fig 3.18B). Bcl6 protein is only observed at 72 hours (Fig 3.3). The significance of the expression of Bcl6 mRNA prior to immunisation remains obscure, but data presented in chapter 4 suggest that it does not mark cells that are only able to become GC cells on activation.

**3.5. IRF4 expression throughout the response to 30μg NP-Ficoll**

IRF4 protein is associated with production of plasmablasts from both the GC and extrafollicular response and is implicated in the induction of CSR; for these processes do not occur in mice deficient in IRF4 (Mittrücker, *et al,* 1997; Klein, *et al*, 2006). It has been suggested that the induction of low level expression of IRF4 protein may drive B cells into CSR through activation of AID, while higher expression levels induce AFC formation (Sciammas, *et al*, 2006). Apart from a few cells – perhaps committed to AFC differentiation, IRF4 is not expressed in GC B cells (Falini, *et al*, 2000) and this is confirmed in the GC present at 72 hours (Fig 3.19). These data suggest that IRF4 expression might be a factor in determining whether B cells recruited into antibody responses differentiate into GC cells or form plasmablasts in an extrafollicular response.

*3.5.1 IRF4 expression in the NP binding cells during the first day of their response to NP-Ficoll.*

In non immunized mice immunohistology reveals that IRF4 protein is expressed in some cells in the red pulp and extrafollicular foci. The location of these cells is consistent with them being background plasma cells (Fig 3.20A and B). Importantly there are only very occasional $IRF4^+$ cells in the MZ and follicles (Fig 3.20B). The number of these $IRF4^+$ cells is far lower than the numbers of NP-specific cells in either the follicles or MZ. Eight hours after immunization immunohistology shows that IRF4 protein is expressed in most if not all NP binding cells in the T zone (Fig 3.20C and D). Some NP-specific B cells are still in the MZ at 8 hours and these are also $IRF4^+$. These $IRF4^+$ $NP^+$ MZ cells probably are about to migrate to the T zone, for the MZ is clear of NP-specific or $IRF4^+$ cells at 24 hours (not shown). The upregulation of IRF4 within 6 hours of B cell activation *in vitro* has previously been reported in response to either BCR ligation (Matsuyama, *et al*, 1995) or CD40 ligation in the presence of IL4 (Gupta, *et al*, 1999).

The immunohistological studies indicate that at best IRF4 protein expression is low in mature recirculating and MZ B cells that have not been recruited into antibody responses and that this is upregulated in most, if not all, cells within 8 hours of being recruited into the response to NP-Ficoll. These studies do not quantify the increases in IRF4 expression and the proportions of cells upregulating IRF4. To obtain quantification the expression of IRF4 was assessed in permiabilized NP-specific B cells by flow cytometry. Prior to immunization FACS analysis shows that most NP-binding cells express levels of IRF4 that are within the 95 percentile staining for the secondary alone control (Fig, 3.21). In some non-immunized mice a

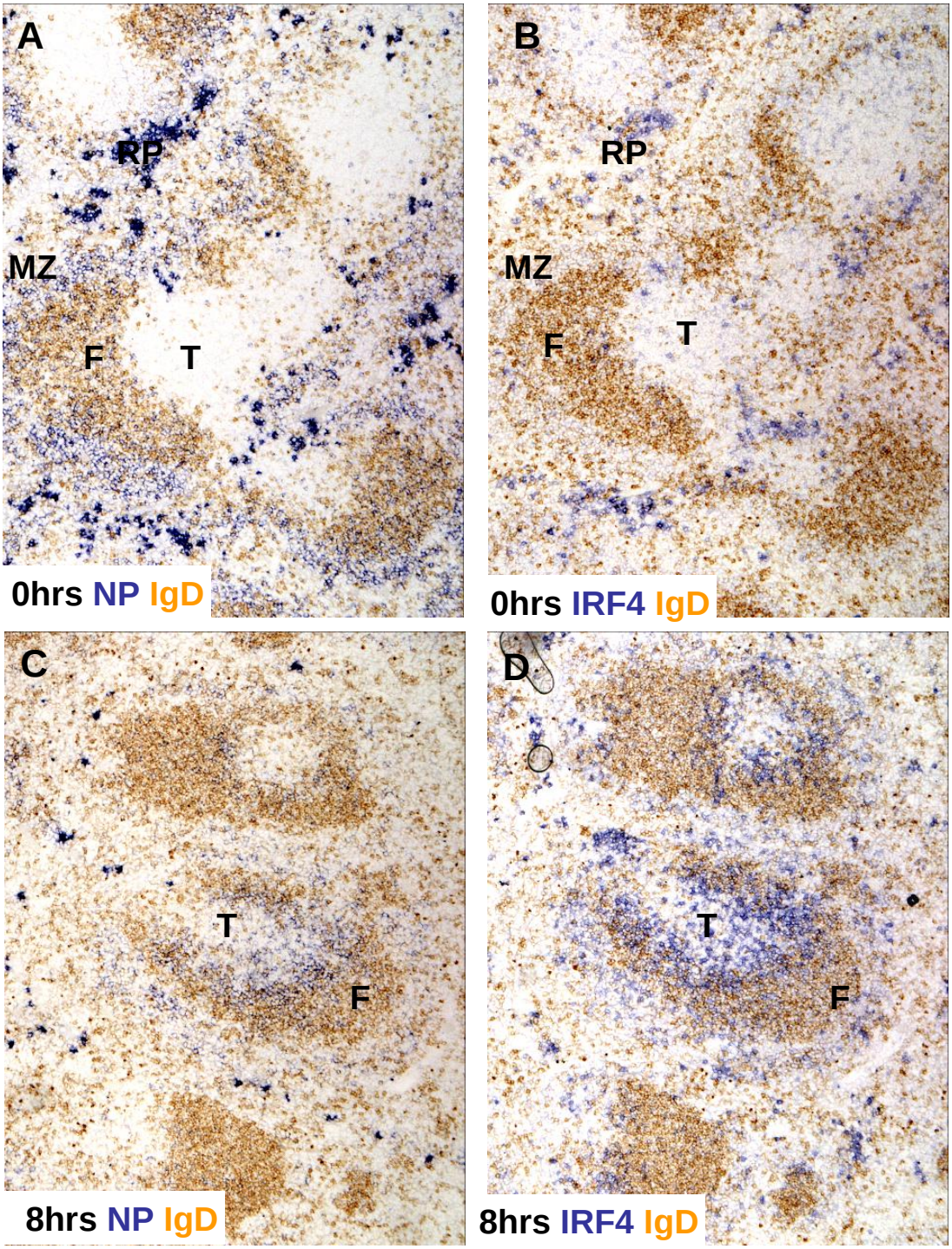


Figure 3.20: IRF4 expression in NP binding cells 8 hours after NP-Ficoll

**A.** NP binding cells in the marginal zone (MS) of the white pulp prior to immunization. **B.** IRF4$^{+}$ cells prior to immunization in extra follicular and red pulp (RP) plasmablasts. **C.** NP$^{+}$ cells at the T zone follicle boundary 8 hours after immunzation. **D.** IRF4$^{+}$ cells at the T zone/follicle boundary 8 hours after immunization. Colours as indicated. F= follicle, T= T zone.

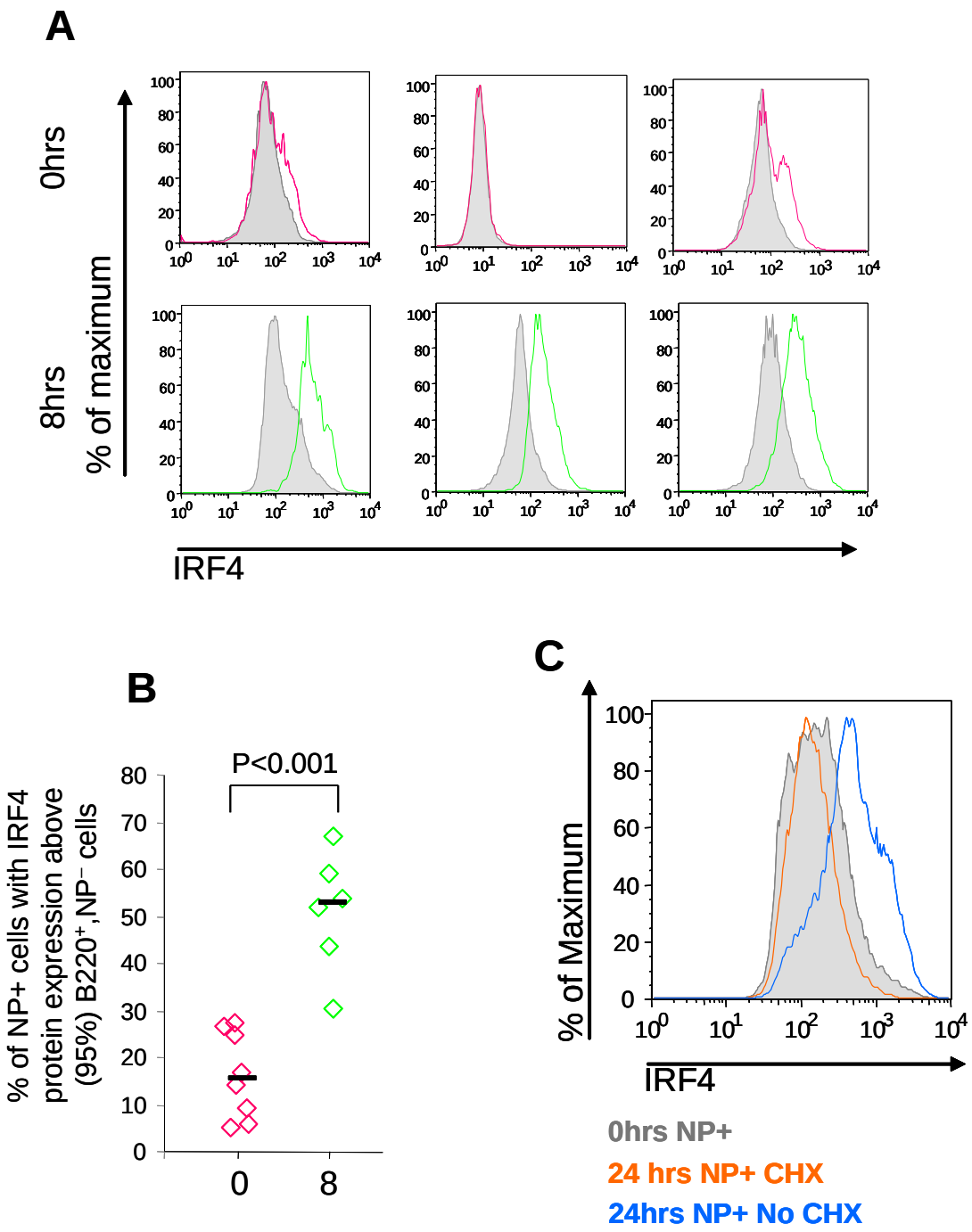


Figure 3.21: IRF4 protein is present prior to immunization but increases after NP-Ficoll immunization

**A.** IRF4 protein prior to and 8 hours after immunisation in NP binding cells. Before immunization a variable but always small population of IRF4 expressing cells can be seen. After immunization IRF4 the amount of IRF4$^{+}$ cells increases. Graphs are representative of 8 pre-immunization and 6 post immunization mice. **B**. The percentage of NP binding cells that are IRF4$^{+}$ compared to expression in NP$^{-}$, non responding, cells. As is detailed in the methods the IRF-4 staining by the majority of NP$^{-}$, B220$^{+}$ cells conforms with the isotype control staining and provides a good internal negative control in the FACS analysis. Bars represent median values. These groups are significantly different p=0.001, Mann-Whitney U test. **C**. IRF4 expression *in vitro* in response to NP-Fitc-Ficoll with or without cyclohexamide (CHX). The increase in IRF4 expression seen at 24 hours is inhibited by the addition of cyclohexamide.

few of the NP-specific B cells contain some IRF4; these cells are seen as a peak to the right hand side of the curve (Fig 3.21A, top row). The levels of IRF4 expressed by this low proportion of $NP^+$ $B220^+$ cells are clearly lower than those of AFC (compare with 3.23B, green curve). Consequently it seems this low level of IRF4 expression by NP-specific B cells from non-immunized mice does not reflect the background AFC. These NP-specific AFC are almost all $Ki67^-$ mature plasma cells (not shown), which do not express high levels of surface anti-NP antibody. It remains to be determined if the low level IRF4 protein expression in some NP-specific splenic B cells from non-immunized QMxB6 mice is due to a background of activated cells or low level expression by a small, but variable, proportion of non-activated B cells.

After BCR engagement a shift in IRF4 protein expression is seen whereby between 30 and 70% (median 50%) of $NP^+$ cells have levels above that of 95% of NP- cells (Fig 3.21A and B). The median level of staining among these $IRF4^+$ cells is around 3 fold the greater than the negative control, but as mentioned above and considered further in the next section, is well below that by AFC. Preliminary *in vitro* experiments with and without the ribosome inhibiting cyclohexamide indicate that this increase in expression is dependent upon *de novo* production of IRF4 (Fig 3.21C).

In contrast to the increase seen in IRF4 protein the array data for IRF4 mRNA expression did not show an increase in expression between 0 and 24 hours (Fig. 3.6). This would suggest that IRF4 protein expression was regulated at the translational level. This conclusion is not supported by analysis of RT2-PCR for IRF4 by single cells. Levels of IRF4 mRNA prior to and 24 hours after immunization are not significantly different (Fig 3.22), but IRF4 mRNA expression increases during the first 8 hours of the response and is significantly higher than that at 0 and 24 hours, indicating at least a degree of transcriptional regulation of IRF4 protein levels.

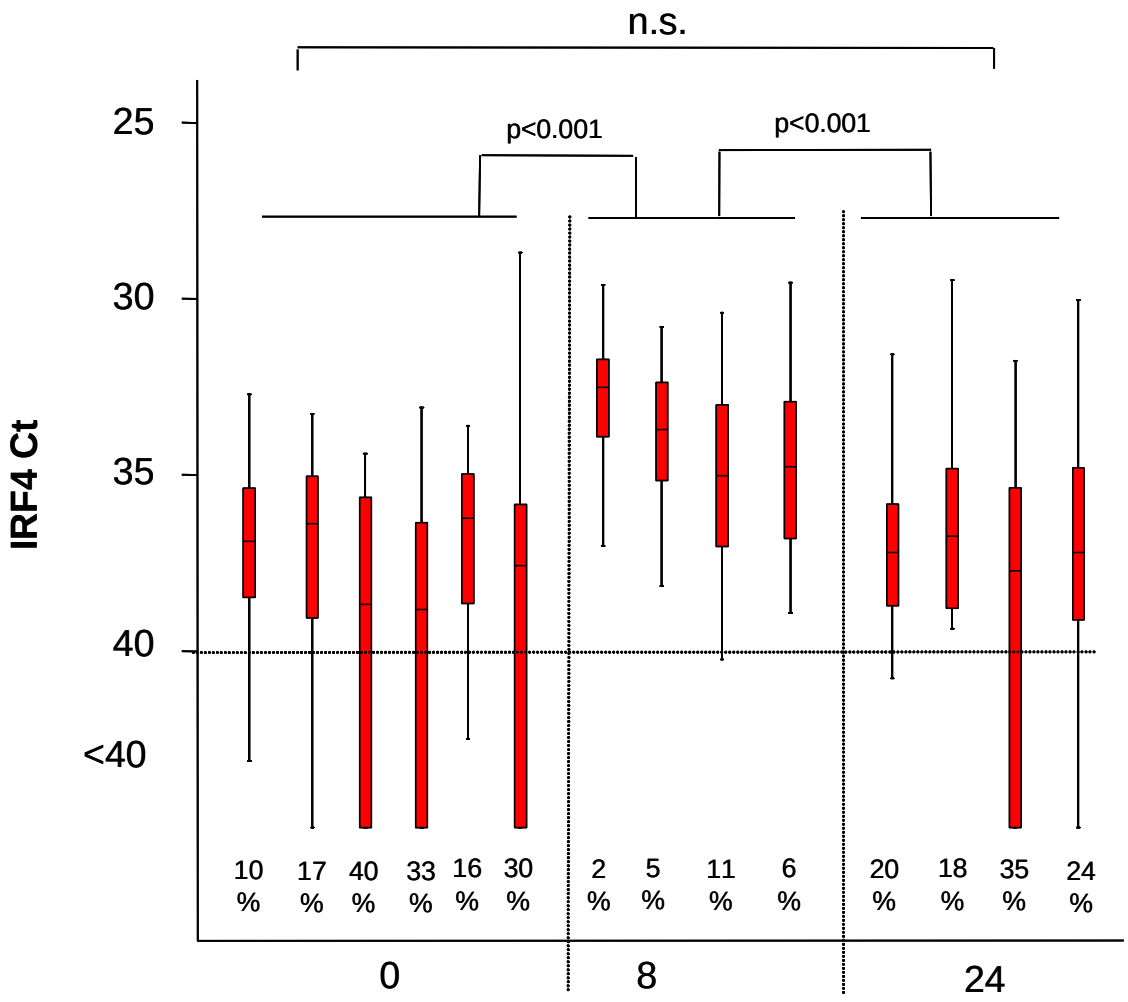


Figure 3.22: IRF4 mRNA expression does not follow IRF4 protein levels during the first 24 hours of the response to 30μg NP-Ficoll.

Box plots to show the expression of IRF4 mRNA in the sorted $NP^+$ $B220^+$ $\beta_2$m positive cells prior to immunization, and at 8 and 24 hours after immunization. Each box is representative of 30-170 cells from one mouse. The centre of the box indicates the median, the ends the quartile range. Whiskers show the 99 percentile. Cells with an IRF4 ct less than 40 are treated as negative. The percentage of cells that are negative is indicated under each plot.

*3.5.2. IRF4 expression in relation to the formation of plasmablasts,germinal centre cells and switching as assessed by co-expression of AID and Bcl-6 on the second and third days of the response*

At 72 hours three populations can be distinguished by flow cytometry in $NP^+$ cells (Fig 3.23.A) i) a distinct IRF4 high population which is $CD138^+$ (Fig 3.23B), ii) an IRF4 low/negative population which is $CD138^-$, and iii) the persisting IRF4 intermediate, $CD138^-$ population that emerged at 8 hours.

*Plasmablast formation:* The IRF4 high, $CD138^+$ population is first apparent at 48 hours (Fig.3.23C) and constitutes some 20% of the responding cells by 72 hours. This is coincident with the emergence of $IRF4^+$ $NP^+$ plasmablasts that are visible by immunohistology in the red pulp in small numbers at 48 hours and fill large areas of red pulp by 72 hours (Fig 3.19). The proportion of responding cells that are $NP^+$ IRF4 high at 72 hours is slightly higher than the proportion of $NP^+$ $CD138^+$ cells (Fig 3.4B). This is consistent with the concept that the upregulation of IRF4 to high levels precedes the expression of CD138. This was previously suggested by Falini, *et al*, (2000). Further evidence that is consistent with this concept is provided by single cell RT2-PCR of sorted populations of $NP^+$ cells at 48 hours after NP-Ficoll (Figure 3.24). In this figure it can be seen that the $CD138^+$ $NP^+$ cells at 72 hours have very few $IRF4^{low/-}$ cells and a substantial proportion of $IRF4^{high}$ cells. These $IFR4^{high}$ cells, which have ct values of fewer than 31 cycles, are boxed in Figure 3.24. Cells with these high levels of IRF4 mRNA are also seen in the $NP^+$ cells sorted at 48 hours, but are very rare in the $NP^+$ $CD138^+$populations sorted at 72 hours. It is plausible that these cells at 48 hours with high levels of IRF4 mRNA are AFC precursors.

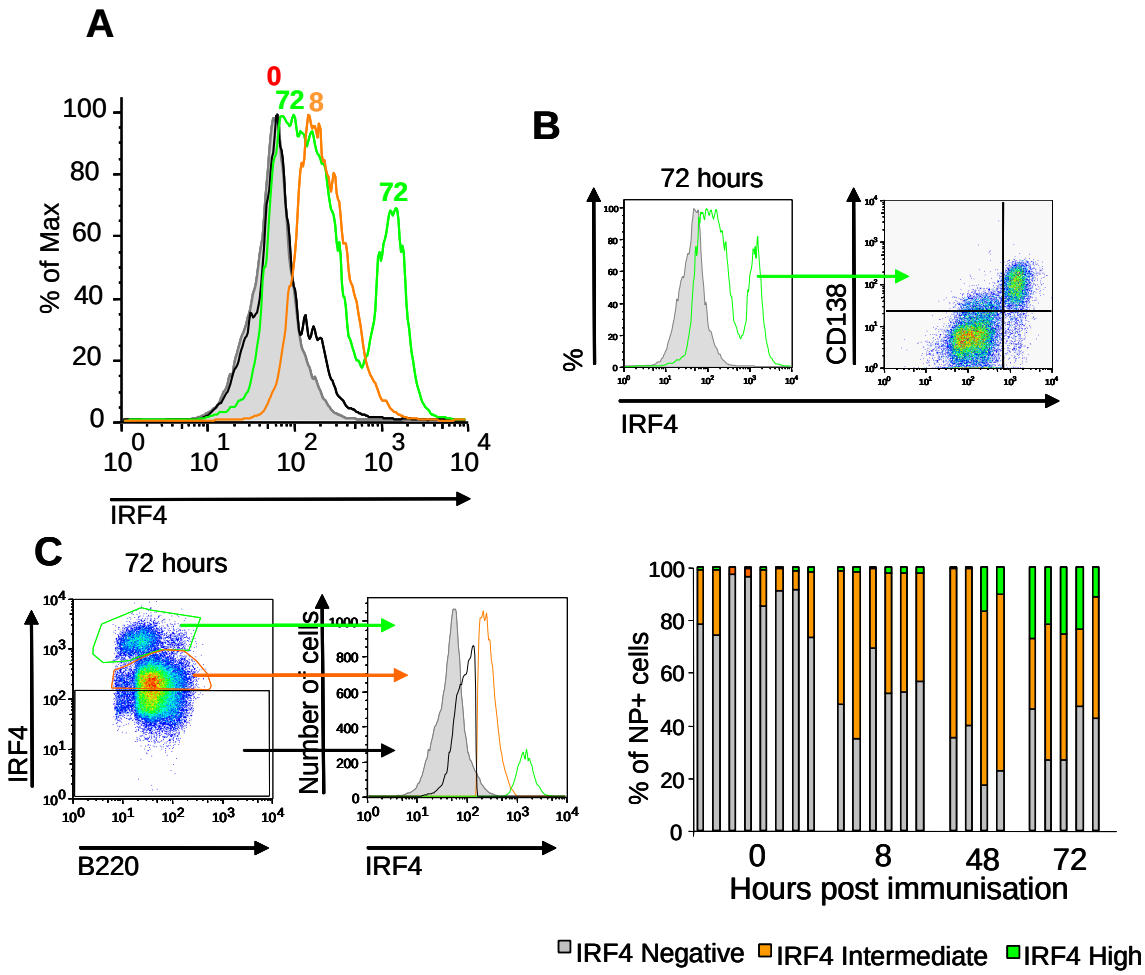


Figure 3.23: IRF4 expression by FACS in NP binding cells after immunization.

**A**. IRF4 expression in NP binding cells prior to immunization and at 8, and 72 hours. Non immunized NP$^-$ B220$^{high}$ cells are grey, NP$^+$ cells prior to immunization are red. Responding cells at 8 hours are orange, and at 72 hours are green. Most NP binding cells have upregulated IRF4 at 8 hours. By 72 hours an IRF4 high and negative population is seen as well as some intermediate cells. **B.** NP$^+$ IRF4 high cells at 72 hours are mostly CD138$^+$ whilst IRF4 low cells are CD138-. Left hand graph shows expression of IRF4 in NP$^+$ cells at 72 hours. Grey curve shows IRF4 expression of NP-B220$^+$ cells. Right hand graph shows the IRF4 and CD138 expression of NP$^+$ cells. **C.** The % of NP$^+$ cells that are IRF4 high (green), IRF4 intermediate (orange) and IRF4 low (grey). An IRF4 intermediate population persists from 8 hours IRF4 high cells, that are CD138 positive, emerge between 48 and 72 hours. IRF4 low cells are within the IRF4 expression of (95%) NP-B220$^{high}$ cells. IRF4 high cells are defined by their clear IRF4 high B220 intermediate phenotype.

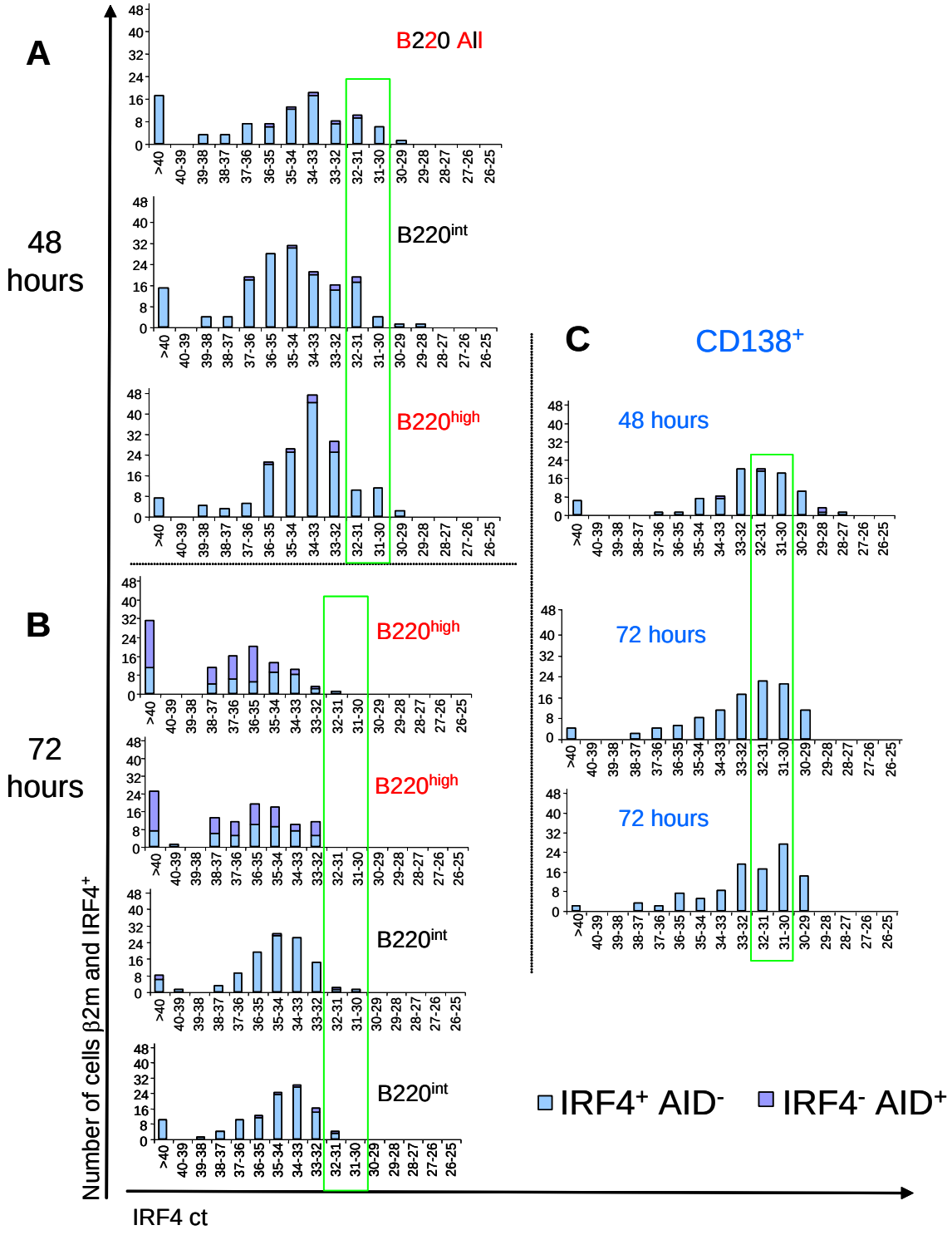


Figure 3.24: IRF4 expression in responding NP+ populations at 48 and 72 hours

The number of cells that express IRF4 mRNA at the indicated Ct. Cells that are IRF4+ are turquoise and those that are IRF4+AID+ are purple. Cells at 48 hours (**A**) show a greater range of IRF4 expression than the CD138- cells at 72 hours (**B**). CD138+ cells at both times (**C**) show high IRF4 expression but some cells express levels similar to those in B.

*Germinal centre formation:* As mentioned above GC cells do not obviously contain IRF4 protein as detected by immunohistochemistry (Figure 3.19). Is it possible to identify germinal centre precursors as $IRF4^{low/neg}$ cells? The median proportion of $IRF4^{low/neg}$ cells $NP^{+}$ cells decreases after immunisation from 90% to 50% at 8 hours and reaches 29% at 48 hours. By 72 hours the number of IRF4 negative cells has increased to 40%. Among the $B220^{high}$ population this is likely in part to reflect the emergence of $NP^{+}$ $IRF4^{-}$ GC cells, which lack IRF4 protein as assessed by immunohistochemistry (Fig. 3.19). On the other hand, as is exemplified in Fig 3.23C, a substantial proportion of the $IRF4^{low/neg}$ $NP^{+}$, $CD138^{-}$ cells at 72 hours are $B220^{int}$ and this subset does not contain Bcl6/AID double positive GC cells. This result indicates that it is unsafe to conclude that all responding $NP^{+}$ cells which are IRF4 protein low/neg are GC precursor cells. The same conclusion has to be drawn from the histograms of single cells IRF4 mRNA expression (Fig 3.24B). Overall the data highlight the difficulty in using levels of IRF4 mRNA expression to determine the future fate of $NP^{+}$ B cells recruited into the response to NP-Ficoll. This is shown by the substantial proportion of IRF4 intermediate cells among those cells that already show features of being plasmablasts and GC cells (Fig 3.24B and C). It will be seen that a substantial proportion of the $CD138^{-}$ cells are $AID^{+}$ and that these divide into $IRF4^{+}$ cells and $IRF4^{int}$ cells (Fig 3.25.A and B). Although for technical reasons IRF4, AID and Bcl6 mRNA levels were not assessed in the same cell extrapolation of the proportion of $AID^{+}/Bcl6^{+}$ cells among the $B220^{high}$ $NP^{+}$ population (Fig 3.25C) shows that many of the $AID^{+}/IRF4^{int}$ cells must have also expressed Bcl6.

*Switching:* Earlier it was shown that switching occurs in B blasts before they differentiate to become plasmablasts. As switching is absent from IRF4-deficient B cells (Klein, *et al*, 2006) we assessed the level of IRF4 expression in 48

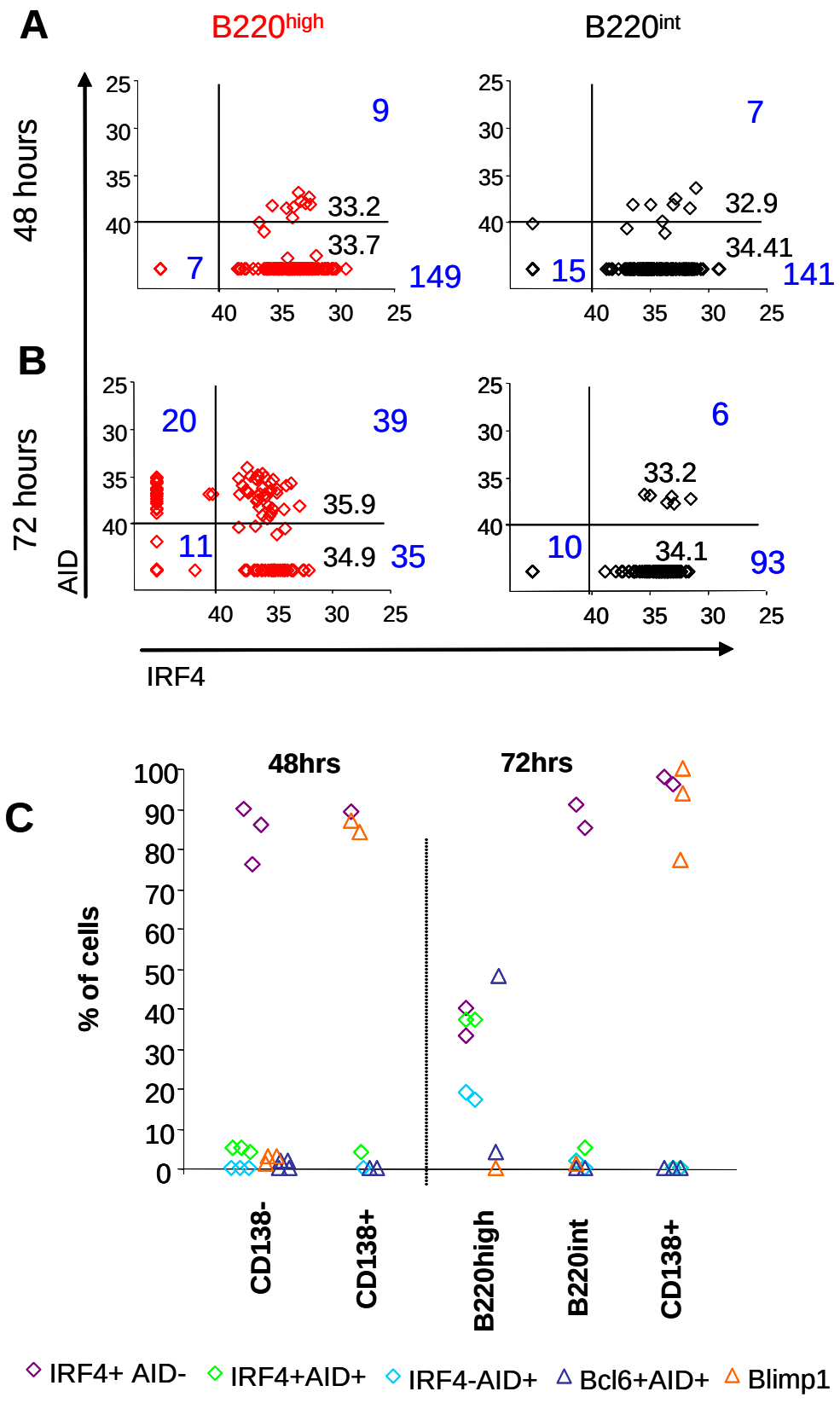


Figure 3.25: IRF4 and AID mRNA are co-expressed in CD138$^{-}$ cells at 48 and 72 hours

Representative plots showing IRF4 and AID mRNA expression in single cells from the NP$^{+}$ CD138$^{-}$ B220$^{high}$ and CD138$^{-}$ B220$^{int}$ cells at 48 (**A**) and 72 (**B**) hours after immunization. Each diamond represents one cell. Cell numbers are in blue and median IRF4 values are in black. **C.** Combined data to show the percentage of cells that express IRF4 alone (blue diamonds), AID alone (red diamonds) or both (green diamonds) in all sorted populations. The percentage of Bcl6$^{+}$AID$^{+}$ cells are indicated by blue triangles and Blimp1$^{+}$ by orange triangles.

hour $NP^+$ B blasts that express AID. These results are shown in Figure 3.25. There are many $AID^-$ cells that express similar intermediate levels of IRF4 mRNA indicating that this level of expression is not exclusively associated with switching cells. It will be seen that all of the $AID^+$ cells express intermediate levels of IRF4 mRNA. It will be noted from these figures that the level of AID expression in the GC cells at 72 hours is generally higher than the level in 48 hour B blasts. This is confirmed in the earlier studies of AID and Bcl-6 expression (Figure 3.18)

## 3.6. Discussion

### *3.6.1 Class switching in the plasmablast response*

$CD138^+$ $Blimp1^+$ cells in the response to NP-Ficoll do not express AID yet switched $IgG_3$ transcripts are expressed in all $NP^+$ populations at 72 hours. As AID has been reported to be essential for class switching to occur (Muramatsu, *et al*, 2000) it would be logical to assume that these plasmablasts can no longer undergo CSR and that this process must have occurred prior to their differentiation. Nevertheless there has been a recent report that CSR may occur in the absence of AID (Arudchandran, *et al*, 2008). At 48 hours, whilst the responding B cells are in the T zone and MZ, $AID^+$ cells are seen in both $CD138^-$ populations co-current with the first expression of switched transcripts. IRF4 has been linked to AID upregulation and class switching in B cells (Klein, *et al*, 2006; Sciammas, *et al*, 2006) and all $AID^+$ cells at 48 hours were $IRF4^+$ at the mRNA level. At 48 hours when $AID^+$, $Bcl6^+$ GC cells have not yet emerged there no obvious or even statistical correlation between IRF4 mRNA levels and AID expression. How CSR is induced on the second day remains to be determined. Strong BCR ligation has been shown to provoke CSR alone *in vitro* (Deenick, *et al*, 1999) however additional stimuli may be required for

the process to occur. Both $CD138^-$ populations at 48 hours express TACI and BAFF-R (Fig 3.7); when these receptors are engaged they can transmit signals that promote CSR (Castigli, *et al*, 2005; von Bulow, *et al*, 2001) although TI-2 responses are independent of BAFF-R (Shulga-Morskaya, *et al*, 2004). Interleukins such as Il-4 (Cunningham, *et al*, 2004) and IFN1 (Le Bon, *et al*, 2001) might also provide additional stimuli for CSR.

### *3.6.2 The hierarchy of IRF4, Blimp1 and CD138 expression in plasmablast development*

It is clear that we have identified the $NP^+$ plasmablast population as $CD138^+$ $IRF4^{high}$, $Blimp1^+$ cells observed in small numbers at 48 hours and frequent at 72 hours (Sections 3.2, 3.3.2, 3.4.2 and 3.5.2). However, it was hoped that in the process of these studies precursors to these cells would be identified. Examination of IRF4 mRNA expression of $CD138^-$ NP-binding cells at 48 hours identified an IRF4high subset that was no longer present among the $CD138^-$ NP-binding cells at 72 hours. It is possible that this $IRF4^{high}$ subset are cells that are about to differentiate into plasmablasts and this is an early indicator of plasma cell differentiation (Section 3.5.2), although this assumes that higher levels of IRF4 mRNA expression preclude protein down regulation. This is in agreement with the published literature on IRF4; IRF4 expression has been shown to up regulate Blimp1 and CD138 (Teng, *et al*, 2007; Sciammas, *et al*, 2006). Our results suggest a hierarchy of CD138, Blimp1 and IRF4 expression as cells differentiate towards plasmablasts. As previously suggested (Section 3.4.2), the presence of Blimp1 negative $CD138^+$ cells may indicate that CD138 is upregulated prior to Blimp1 expression. However, the relationship between CD138 and IRF4 is more

complicated. Not all $CD138^+$ cells express IRF4 mRNA (Fig 3.23B), yet IRF4 protein is expressed prior to CD138 in GC cells differentiating into AFC (fig 3.19A; Falini, *et al*, 2000). Whilst IRF4 is necessary for plasma cell generation (Klein, *et al*, 2006) it has been reported that IRF4 is not always expressed in $CD138^+$ plasma cells, and vice versa (Falini, *et al*, 2000). Perhaps differentiated plasma cells do not require continuous IRF4 protein expression. The expression of these three plasmablast markers will be examined further in the following chapters.

*3.6.3. The identification of germinal centre cells and their precursors*

Despite the limitations of using bulk sorts the array analysis indicated that GC cells were contained within the $B220^{high}$ population at 72 hours (Sections 3.3.3 and 3.4.3). Single cell RT2-PCR identified these cells as Bcl6, AID double-positive cells. These cells were not present at 48 hours and this finding correlates with the immunohistology, which fails to identify clusters of cells expressing Bcl6 protein in follicle centres before 72 hours. By 72 hours the $B220^{high}$ subset has selectively upregulated Bcl6, FAS, GCET2, RAPGAF1 and BAFFR which are all associated with GC B cells, but the array data fail to show upregulation of any of these mRNAs between 24 and 48 hours. The exception to this trend is AID, but it can be argued that the low-level AID expressed in a significant minority of cells at 48 hours reflects switching activity. Thus although the results obtained point to candidate AFC precursors at 48 hours they fail to identify cells that are about to become GC B cells. The process of selection following somatic hypermutation in GC requires antigen recognition through the BCR and subsequent cognate interaction with CD4 T cells (Liu, *et al*, 1989; and Maclennan, 1994). BCR expression is maintained through the continued expression of Pax-5 (Fukuda, *et al*, 1997; Lin, *et al*, 2002), while the

production of Bcl6 protein represses the expression of genes that promote differentiation to AFC (Reljic, *et al*, 2000). The decline in IRF4 expression in GC cells does not appear to induce Bcl6 expression (Section 3.5.2). However, excess Bcl6 protein has no effect on IRF4 activity *in vitro* (Teng, *et al*, 2007) so the downregulation of IRF4 may be induced through some other mechanism. A model in which strong GC responses are induced with few cells becoming AFC outside GC is used to search for the phenotype of GC B cell precursors in chapter 5.

### *3.6.4 The role and origins of the CD138$^{+}$ B220 intermediate population*

A large NP$^{+}$ B220 intermediate, CD138 negative population emerges at 48 hours (section 3.2). Their B220 phenotype might suggest a B1b population (Montecino-Rodriguez, *et al*, 2006), the differentiation of B cells towards plasmablasts (Sanderson, *et al,* 1989) or the emergence of B cell memory (Driver, *et al*, 2001). There is evidence for all of these events. The gene array data on the CD138$^{-}$ B220$^{int}$ cells at 72 hours shows a few similarities with memory B cells (section 3.3.4; Kuo, *et al*, 2007). However, the nature of this study has meant that a NP$^{+}$ memory population as a result of this immunization protocol has not been confirmed. B1b cells participate in responses to TI-2 antigens (Martin, *et al*, 2001) and have been shown to provide long term memory to NP-Ficoll in chimeric mice (Hsu, *et al*, 2006). Most of the NP$^{+}$ B cells in the spleen of non-immunized mice are MZ B cells and these are likely to be the major contributors to the early response to NP-Ficoll. Nevertheless, it is possible that B1b cells are recruited into the spleen from the periptoneal cavity. The B1b response to antigen is swift (Itakura, *et al*, 2005) and it been reported that B1 cells express Blimp1 protein within 28 hours of stimulation, far quicker than B2 cells (Fairfax, *et al*, 2007). If this is the case then it

seems unlikely that the large B220 intermediate population that has emerged by 48 after 30μg NP-Ficoll is comprised of responding B1b cells for they do not express Blimp-1 mRNA. Further analysis of B1b cell markers by FACS may help identify B1 and B2 cells that are present in already identified populations and confirm the presence of B1 cells. There is also the possibility that these cells are still in the process of differentiation. Indeed, the $CD138^{+}$ $B220^{int}$ population is most closely related to the $CD138^{-}$ $B220^{int}$ population according to TMEV analysis (Fig 3.5), which might imply that these cells are differentiating towards AFC. The large number of cells that participate in the response could mean that the factors associated with AFC development such as cell-cell contact or interleukin/chemokine receptor binding (Gao, *et al*, 2008; Kim, *et al*, 2008) are diluted and consequently the process is delayed, or never fully induced.

# Chapter 4:

# Varying the proportion of cells becoming plasmablasts and switching in the extrafollicular response to study molecules influencing these processes

The response to 30μg NP-Ficoll produces both a large extrafollicular plasmablast response and germinal centres (GC). To study further the roles of Bcl6, Blimp1, AID and IRF4 in class switch recombination (CSR), and the induction of B blast differentiation to plasmablasts or GC B cells, new immunization protocols were devised that alter the proportions of NP-specific cells differentiating in these different ways. In addition, it was hoped that these variations in the response could be used as a means to identify other molecules that are involved in directing differentiation. Three models were selected and the relative impact on follicular and extrafollicular responses and class switching of the first two of these is shown in Table 4.1.

i) To provoke an antibody forming cell (AFC) response without class switching and little GC formation a low dose of NP-Ficoll was administered to the QMxB6 mouse. This protocol has previously been reported to selectively induce an extrafollicular response without GC (Garcia de Vinuesa, *et al*, 2000). In the event, the switch off of GC was relative rather than absolute, but the low dose immunization has provided an extrafollicular response with little or no switching.

ii) Higher levels of class switching were induced through the co-administration of PolyI:C and 30μg NP-Ficoll. This manipulation was based on previous studies by Le Bon *et al* (2001).

iii) A strong NP-specific GC response with few NP-specific plasmablasts was induced using the carrier primed system (Toellner, *et al*, 1996). In this system congenic wild type C57Bl6 mice were primed with chicken gamma globulin (CGG). Five weeks later these mice received QM B cells *i.v.* followed by challenge with soluble NP-CGG *i.p.* (section 2.2). As the carrier primed response in QM B cell-reconstituted C57Bl6 mice is not directly comparable to those invoked in CGG-primed QMxB6 mice, control chimeras were immunized with 30μg NP-Ficoll. These experiments in carrier primed chimeras are reported in Chapter 5.

| | Cells per $mm^2$ at 96hours that had switched to IgG3 | % intercepts that fall on extrafollicular plasmablasts at 96 hours | % intercepts that fall on germinal centre cells at 96 hours |
|---|---|---|---|
| 30μg dose NP-Ficoll in QMxB6 | 28% | 19% | 6% |
| 1μg dose NP-Ficoll in QMxB6 | <1% | 4% | 1% |
| 30μg dose NP-Ficoll + poly I:C. in QMxB6 | 38% | 9% | 6% |

Table 4.1: The proportions of NP-specific cells in the three QMxB6 immunization protocols that at 96 hours after immunization (a) had undergone switch recombination to IgG3, (b) had entered germinal centres, or (c) had become extrafollicular plasmblasts.

**4.1. A low dose of NP-Ficoll induces B blasts and by 96 hours has induced plasmablasts but these have not undergone class switch recombination and only small or no germinal centres are formed.**

It has previously been reported that by altering the dose of NP-Ficoll administered to QMxB6 mice the proportions of GC and extrafollicular AFC generated can be changed (Garcia de Vinuesa, *et al*, 2000). As the dose of NP-Ficoll decreases the area occupied by NP specific GC declines until only the extrafollicular plasmablast response remains. This report did not mention the affect of NP-Ficoll administration on CSR. However, we observed that immunization with 1μg NP-Ficoll induced a plasmablast response without a detectable number of these plasmablasts switching Ig class, as will be described later in this chapter (Section 4.1.2). This provided an opportunity to test a number of hypotheses relating to CSR that were generated from the results of single cell RT-PCR described in chapter 3. First, the $AID^+$ cells observed at 48 hours in the response to 30μg NP-Ficoll might be plasmablast precursors undergoing CSR, or GC precursor cells. These are not necessarily mutually exclusive possibilities. As both switching and somatic hypermutation in GC B cells require AID it is anticipated that the response of QMxB6 mice following immunization with 1μg NP-Ficoll would be unlikely to be associated with AID positive B blasts at 48 hours after immunization. Second, as GC formation is greatly reduced, is the proportion of B Blasts expressing Bcl6 mRNA at 48 hours reduced? This might be expected if those B cells from non-immunized mice that express Bcl-6 mRNA are predestined to become GC forming cells. Third, as most B blasts become plasmablasts in response to 1μg NP-Ficoll do these B blasts have a

higher average level of IRF4 expression than is seen following immunization with 30μg NP-Ficoll? In the latter case as both plasmablasts and GC are formed one might expect to see a mixture of $IRF4^{high}$ and $IRF4^{low}$ B blasts at 48 hours. Fourth, is there a loss of a subset of B blasts with distinctive IRF4 expression that are about to undergo CSR? To try to answer the above questions B blasts were studied 48 hours after immunization as this is the time immediately before the point when most Blimp-$1^+$ plasmablasts or $AID^+$, Bcl-$6^+$, GC B cells emerge (Figure 3.18; Figure 3.19).

A titration of NP-Ficoll was performed in QMxB6 mice and the GC and plasmablast responses were counted at 96 hours (Fig 4.1.A); this is the peak of the GC response to NP-Ficoll and is before they undergo involution (Garcia de Vinuesa, *et al*, 2000). The results obtained broadly confirm the findings of Garcia de Vinuesa, *et al*¸ 2000 – counts from immunohistology showed the spleen area occupied by $NP^+$ GC and AFC decreased as the amount of NP-Ficoll was reduced (Fig. 4.1A). When immunized with 1μg NP-Ficoll there was still an appreciable plasmablast response but very little in the way of GC in 2 of 3 mice (Fig 4.1A). The plasmablast response at this dose was more marked than can be deduced by counting the total number of AFC (Fig 4.1.B). The appreciable background number of AFC in non-immunized mice are predominantly Ki67 plasma cells, while the AFC in the mice immunized with 1μg NP-Ficoll were $Ki67^+$ plasmablasts (Fig 4.1.C and 4.2). This indicates that there was a definite plasmablast response even if this was smaller than that induced with 30μg NP-Ficoll. The data from Garcia de Vinuesa, *et al*, (2000), show that no GC were formed in response to 1μg NP-Ficoll, however, our data do show that in 1 of 3 mice small but definite GC formed. Importantly, as indicated in the previous section, in contrast to the response to 30μg NP-Ficoll none of the plasmablasts from mice 96 hours after immunization with 1μg NP-Ficoll had undergone CSR to $IgG_3$ (Fig. 4.1D).

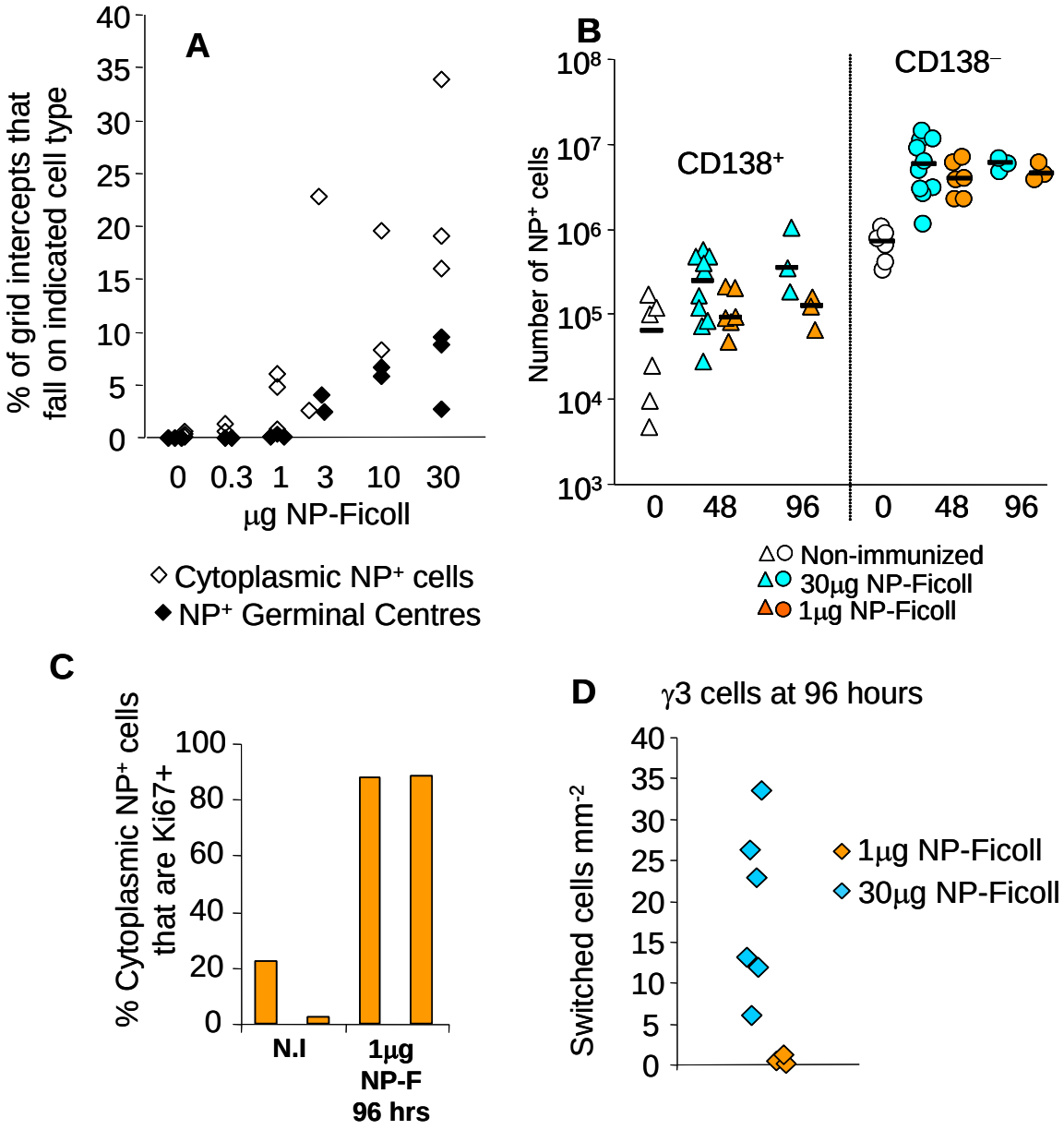


Figure 4.1: Low dose NP-Ficoll produces an extrafollicular plasmablast response with minimal germinal centre formation or Ig class switching

**A**. The 1μg response to NP-Ficoll results in plasmablasts (open diamonds) but very few germinal centres (closed diamonds). **B**. Both the CD138$^+$ and CD138$^-$ populations expand in response to 1μg NP-Ficoll (orange symbols) but less than that seen in the 30μg response (blue symbols). **C**. The cells that stain for cytoplasmic NP are mostly Ki67$^-$ prior to immunization but are KI67$^+$ plasmablasts at 96 hours after 1μg NP-Ficoll. Photomicrographs showing this are presented in Figure 4.2. **D**. At 96 hours very little, if any class switching has occurred in response to 1μg NP-Ficoll (orange diamonds) compared to 30μg NP-Ficoll (blue diamonds).

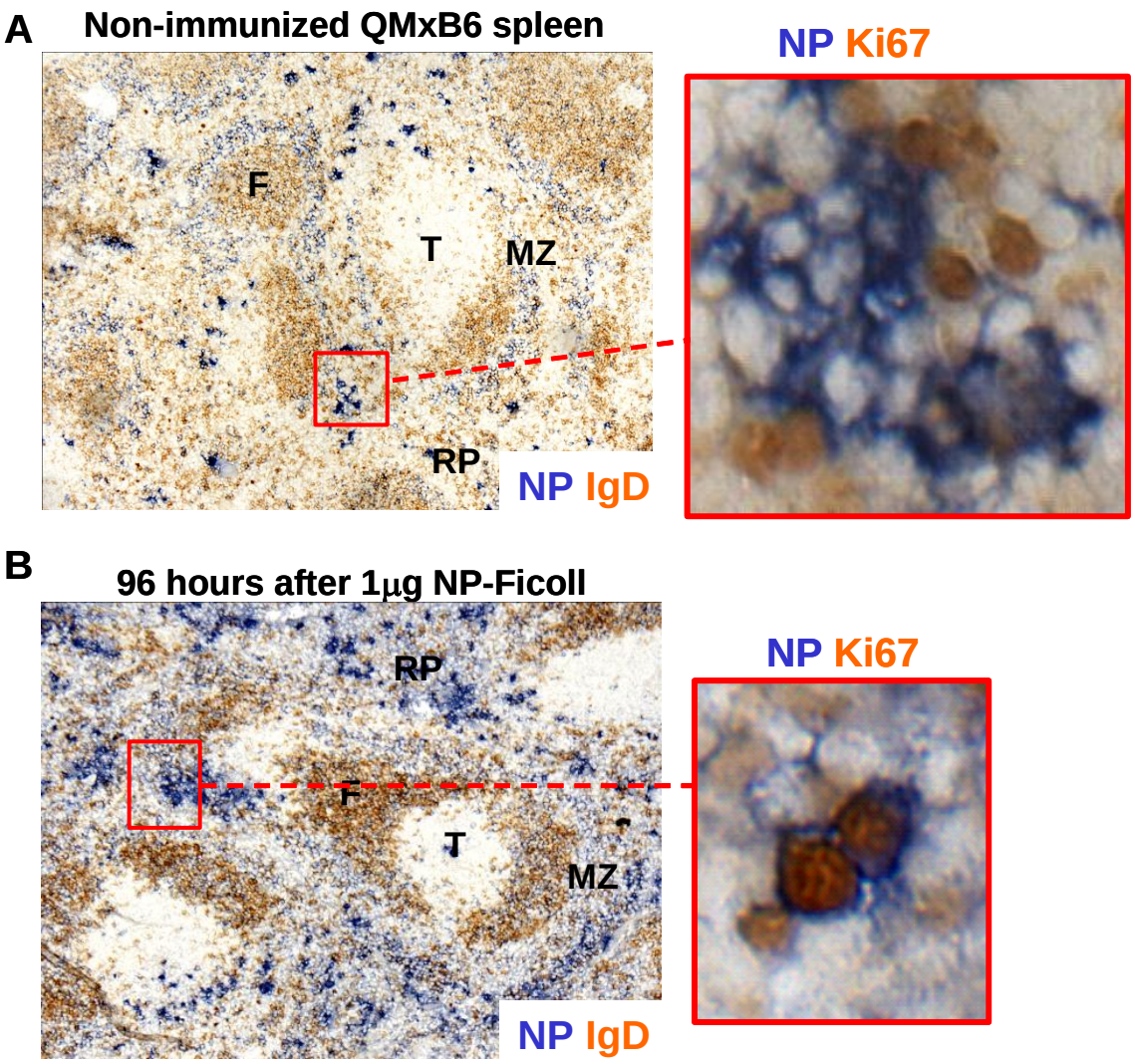


Figure 4.2: Plasmablasts are present at 96 hours in response to 1μg NP-Ficoll.

**A**. In the non-immunized QMxB6 spleen the $NP^+$ AFC present in the red pulp (RP) and these are mostly $Ki67^-$. The majority of NP binding B cells are in the marginal zone (MZ). **B**. 96 hours after 1μg NP-Ficoll there has been an increase in the plasmablasts in the red pulp and these are mostly $Ki67^+$. $NP^+$, $Ki67^+$ cells have blue cytoplasm and a brown nucleus. $NP^+$, $Ki67^-$ cells have blue cytoplasm and a clear nucleus. T = T zone/ PALS. IgD or Ki67 in brown and NP in blue.

*4.1.1 The characteristics of the B blasts located in the T zone at 48 hours after 1μg NP-Ficoll*

Forty eight hours after immunization with 1μg NP-Ficoll $NP^+$ B blasts were located at the boundary of the T zone and the follicle. These stain positive with the antibody Ki67, which binds to intercellular proteins expressed during replication, indicating cell division and, on the basis of analysis in serial sections, most of these expressed IRF4 protein, (Fig. 4.3, red boxes). As we observed $NP^+$ plasmablasts at 96 hours in the response to 1μg of NP-Ficoll and a small or absent GC response it would be expected that these B blasts are predominantly plasmablast precursors. Consequently these cells might be expected to show gene and protein expression patterns associated with the plasmablast differentiation pathway. For example, most of the B blasts after 1μg NP-Ficoll might be expected to be $IRF4^{high}$ at the protein and/or mRNA level while those after 30μg NP-Ficoll might be mixtures of $IRF4^{high}$ and $IRF4^{low}$ cells.

There was an appreciable population of $Ki67^-$ $IRF4^-$, $NP^+$ cells present in the marginal zone at 48 hours. These are presumed to be non-responding cells; the smaller B blast response in the 1μg as compared with the 30μg response observed at this time is likely to reflect a reduction in cross linking of BCRs compared to the response to 30μg NP-Ficoll. The presence of a NP-binding, non proliferative population at 48 hours requires that the proportion of these cells should be determined before meaningful analysis of the FACS and RT2 PCR data can occur.

The median number of NP-binding cells 48 hours after 1μg NP-Ficoll was around 5 times greater than the median number prior to immunization (Fig 4.3B). The number of cell divisions the responding cells have completed at this stage is 3,

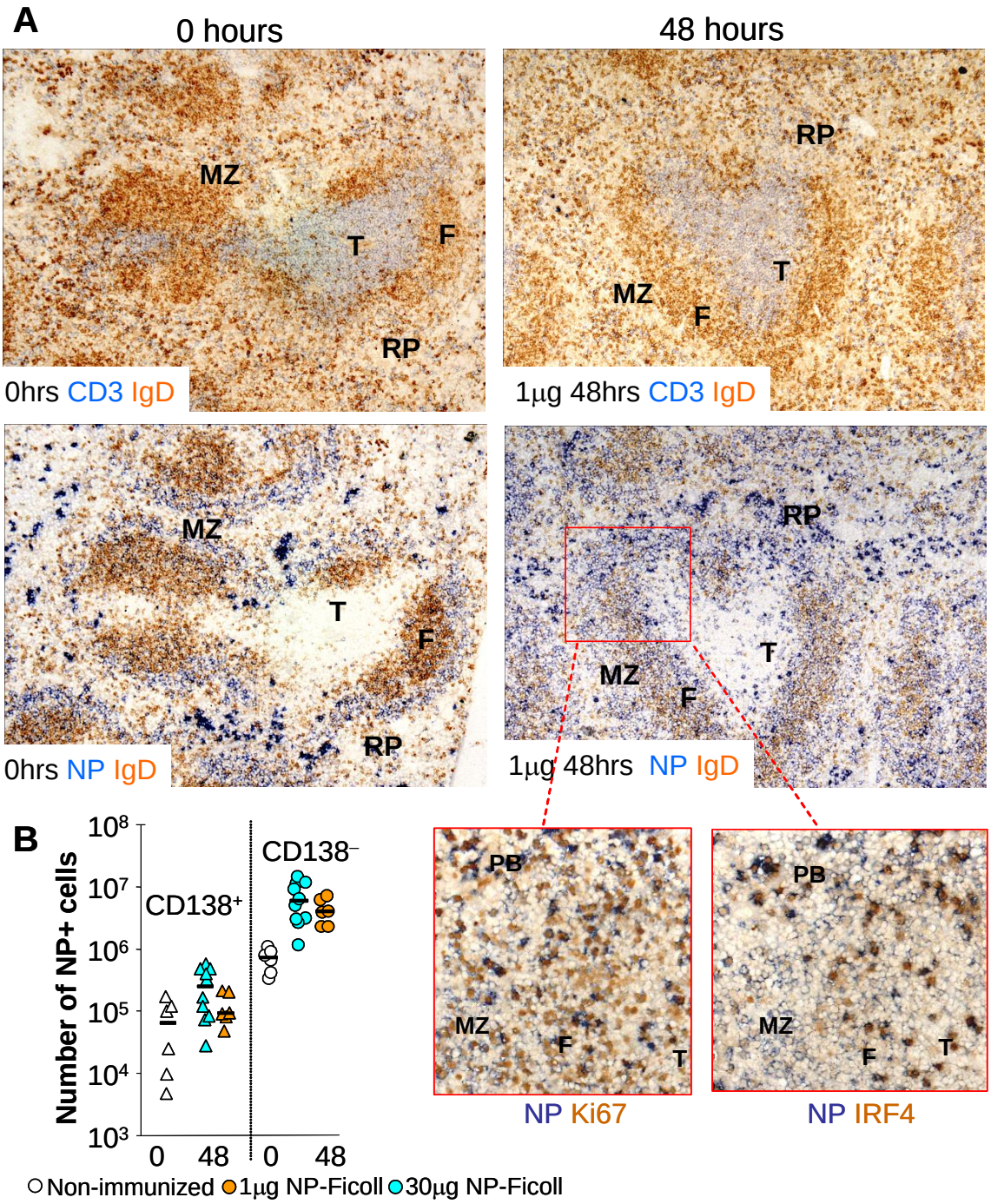


Figure 4.3: At 48 hours the 1μg NP-Ficoll response induces B blasts at the T zone/follicular boundary.

**A**. 48 hours after administration of 1μg NP-Ficoll (right column) NP binding cells are seen at the boundary of the T zone (T) and follicle (F). A number of IRF4$^{high}$, Ki67$^{+}$ plasmablasts are seen in the red pulp (RP) and these are more numerous than those present prior to immunization (left column). The marginal zone (MZ) retains some NP binding cells in the immunized mice and these are IRF4$^{neg}$. **B**. The number of both CD138$^{-}$ and CD138$^{+}$ cells increases between 0 and 48 hours.

as estimated by Hsu (2004) in her analysis of the response to 30μg NP-Ficoll. This is consistent with the observed increase in the number of $NP^+$ cells in the 30μg response between 0 and 48 hours (Fig 4.3). If it is assumed that the same proliferation rate occurs following immunization with 1μg NP-Ficoll it can be calculated that ~40% of the $NP^+$ cells failed to respond and these amount to ~6% of the $NP^+$ cells at 48 hours (the way this was calculated is set out in the methods).

The relative proportion of AFC in the 30μg as opposed to 1μg response can best be deduced from the quantitative immunohistology reported in Fig 4.1A. This is because surface CD138 expression ceases to identify all AFC after 72 hours into the responses to NP-Ficoll. By comparing the median number of graticule intercepts (see methods) falling on cells expressing cytoplasmic anti-NP antibody it can be estimated that there are ~5 times as many AFC in the spleen at 96 hours in the higher NP-Ficoll response than at the same time in the lower-dose response. This contrasts with the number of $CD138^-$, $NP^{high}$ B blasts at 48 hours, which is about 1.4 times higher in the 30μg response (Fig. 4.3.B). These data are consistent with the conclusion that some of the B blasts induced in the response to 1μg NP-Ficoll at 48 hours die without differentiating into plasmablasts. On the basis of the difference in the number of cells in both responses at 48 and 96 hours it can be estimated that some 70% of the B blasts present in the 1μg response at 48 hours will die without becoming plasmablasts (the means used to make this calculation is set out in the methods). This conclusion has to be tentative as the number of observations in the immunohistology was only 3 spleens per group and there is large variation between mice in these small groups. Nevertheless it seems likely that a considerable number

of B blasts die by apoptosis without differentiating into plasmablasts in the lower dose response.

4.1.1.1 The expression of B220 and IRF4 in relation to the activation state of $NP^+$ blasts at 48 hours

FACS analysis of $NP^+$ B cells at 48 hours in response to 1μg compared to 30μg NP-Ficoll is shown in Fig 4.4. Most $NP^+$ cells 48 hours after 1μg NP-Ficoll were $B220^{high}$; in contrast to the 30μg only a tiny $B220^{int}$ population emerged. This implies that most of the plasmablasts that developed on the 3rd and 4th day of the 1μg response came from $B220^{high}$ rather than $B220^{int}$ $NP^+$ B blasts. The size of the $B220^{int}$ population in the two responses is broadly proportional to the number of plasmablasts produced in the response. The data are consistent with the possibility that the $B220^{int}$ cells are plasmablast precursors, but provide no direct evidence to support this conclusion. It cannot be excluded that this population also includes cells that will form GC. The origin and role of the $B220^{int}$, $NP^+$, B blast population was discussed at the end of Chapter 3 and, in the light of its unimpressive size in the 1μg response, is considered further in the discussion to the present chapter.

The expression of IRF4 protein by the $NP^+$ cells at 48 hours after 1μg NP-Ficoll was variable (Fig 4.4B). More than half the cells contained little or no IRF4 protein. The majority of the other cells were $IRF4^{int}$. The small fraction of $IRF4^{high}$ cells in the 1μg response was similar in size to the $CD138^+$ population (Fig 4.4A) and may represent cells that have already differentiated into plasmablasts. $Ki67^+$ $NP^+$ cells with cytoplasmic staining characteristic of plasmablasts and strong IRF4 expression were observed in the red pulp at this stage (Fig 4.3). Taken together the

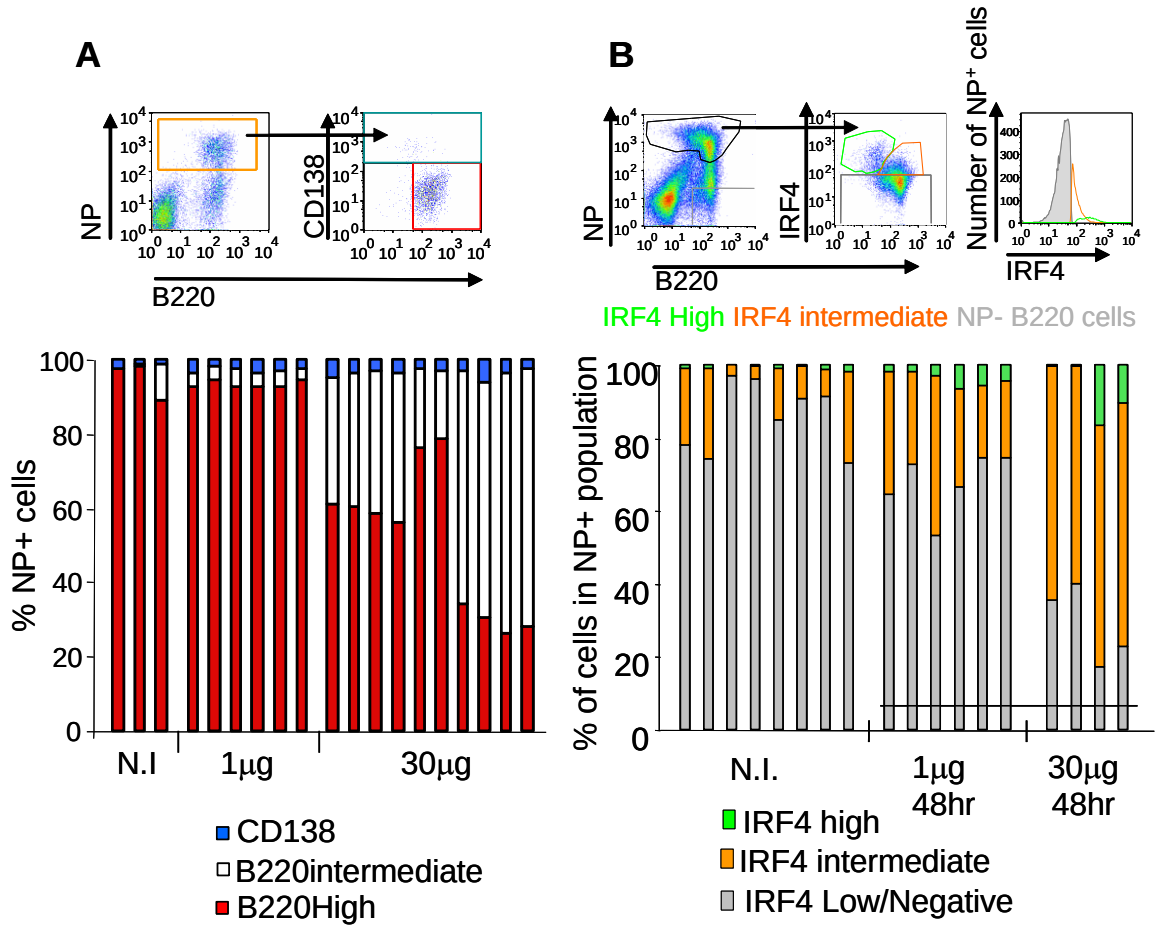


Figure 4.4: The B blast response to 1µg NP-Ficoll consists of a large B220$^{high}$, IRF4$^{neg}$ population

A. FACS plots of lymphocytes gated on NP-PE binding and B220 expression. Plots are typical of the six mice observed. The responding cells are further gated to show CD138 expression. The responding NP$^{+}$ cells are mostly B220$^{high}$ with few B220$^{int}$ and CD138$^{+}$ cells and comparison is made to non-immunized mice and those in the 30µg response at 48 hours. B. The gating of NP$^{+}$ B blasts IRF4 expression is shown, the plot is typical of the six mice observed. The proportion of responding cells that are IRF4$^{neg}$, IRF4$^{int}$ and IRF4$^{high}$ in non immunized QMxB6 mice and the 1µg and 30µg at 48 hours. The horizontal line indicates the percentage of cells that are not participating in the response (6%).

data support the concept that high IRF4 expression is a consequence of plasmablast development and not the initial cause. There are a number of possible reasons for there being fewer IRF4$^{int}$ and more IRF4$^{low/neg}$ NP$^{+}$ cells in the 1μg response compared to the 30μg response. A minority of the IRF4$^{low/neg}$ cells in the 1μg response can be accounted for as being non- responding NP$^{+}$ cells. The previous section identified that a median of some 6% of the NP$^{+}$ cells at 48 hours are non responders. After allowing for the non-responders there is still a larger proportion of IRF4$^{low/neg}$ among the NP$^{+}$ cells in the 1μg response. As this response yields small or no GC it is not possible to link more than a small fraction of these IRF4$^{low/neg}$ cells with GC formation. It could be that these IRF4$^{low/neg}$ cells are B blasts that received only a weak BCR signal with the low does of NP-Ficoll used. These IRF4$^{low/neg}$ B blasts might be the substantial population that dies without making the transition to plasmablasts. In order for B blasts to transition to plasmablasts additional signals are required. This was illustrated through studies of the response to NP-Ficoll in Btk-deficient mice (Vinuesa, *et al*, 2001). This will be considered in the discussion to this chapter. There was a small proportion of IRF4$^{low/neg}$ NP$^{+}$ cells at 48 hours in the 30μg response. These might be one or a combination of the following: GC precursors, non-responding cells or cells that have received a low BCR signal and are destined to die without further differentiation. The data provide no direct evidence for or against any of these possibilities.

As mentioned earlier IRF4 is necessary for CSR (Sciammas, *et al,* 2006). The lack of CSR induced by the 1μg response where a substantial IRF4$^{int}$ population of B blasts is induced shows that low level expression of this protein by itself is not sufficient to induce CSR. In the next section the association between AID expression and switching is tested in the 1μg response.

*4.1.2 A lack of B blasts expressing AID mRNA in the response to 1ug NP-Ficoll is consistent with the observed lack of switching*

At 48 hours in the response to 30μg NP-Ficoll a population of $Bcl6^{-}$ $AID^{+}$ cells was observed (Fig 3.18). Under these conditions we also observed $\gamma_3$ switched transcripts, i.e. processed transcripts of Iμ rearranged to C $\gamma_{3,}$ in some $NP^{+}$ cells (Fig 3.1.B). After 72 hours we also observed the appearance of AFC with cytoplasmic $IgG_3$ (Figures 3.13 & 3.14). In the response to 1μg NP-Ficoll none of the 252 B blasts collected at 48 hours from 2 of 3 mice expressed AID, but in the third mouse 3/159 B blasts (2 $B220^{high}$ and 1 $B220^{int}$) contained AID mRNA. This low proportion of the $NP^{+}$ B blasts that express AID mRNA 48 hours after 1μg NP-Ficoll is consistent with the paucity of switched cells observed at 96 hours (Fig. 4.5.B). As mentioned, low expression of IRF4 has been associated with CSR in B cells (Sciammas, *et al*, 2006). That there is intermediate expression of IRF4 in 25-45% of B blasts at 48 hours indicates that this level of IRF4 expression by responding cells is not sufficient to drive AID expression in the vast majority of these blasts. This is consistent with data from the response to 30μg NP-Ficoll that shows no correlation between IRF4 expression and AID mRNA levels in 48 hour B blasts (Fig 3.25).

*4.1.3. Why do more 48 hour $NP^{+}$ cells express Bcl6 mRNA in the 1μg than the 30μg response?*

The proportion of cells that express Bcl6 mRNA is higher than that observed in response in the 30μg response (Fig 4.5.A). The median number of $NP^{+}$ cells expressing Bcl6 mRNA at 48 hours in response to 1μg is ~19% whilst in the 30μg response it's ~6%. The non responding cells, which comprise ~6% of the $NP^{+}$ cells at 48 hours, can only account for about $1/3^{rd}$ of these cells. Also, it is unlikely that the

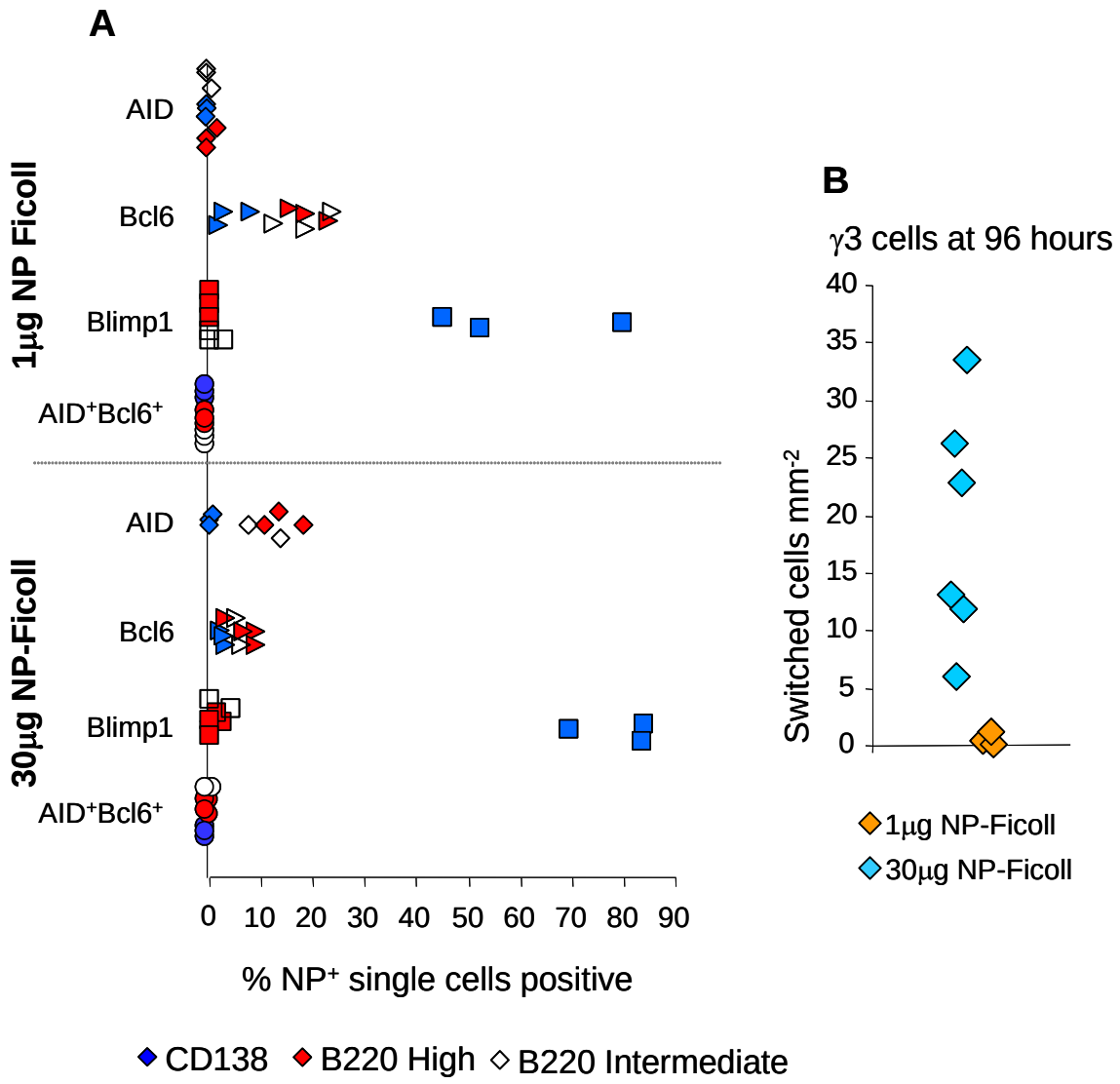


Figure 4.5: Single cell RT2-PCR analysis of NP$^+$ cells shows low AID expression at 48 hours that correlates with reduced class switching at 96 hours.

**A.** The response to 1μg NP-Ficoll shows low expression of AID and Blimp1 but high Bcl6 expression in comparison to the 30μg response. The percentage of cells expressing the genes either singly or together is shown. CD138$^+$ cells are blue, B220$^{high}$ cells red and B220 intermediate cells are white. **B.** The lack of class switch recombination at 96 hours is perhaps a result of the lack of AID expression. The number of switched cells at 96 hours is shown for the 1μg (orange diamonds) and 30μg NP-Ficoll response (blue diamonds).

Bcl6$^{+}$ cells in the B220$^{int}$ population are non-responders as the B220$^{int}$ cells only emerge after immunization. It seems plausible that the responding B220$^{high}$ population might include those cells recruited into the response that disappear, probably by undergoing apoptosis, without differentiating into plasmablasts or GC blasts. Bcl6 mRNA expression was also seen in 3-12% of the CD138$^{+}$ NP$^{+}$ cells 48 hours after 1μg NP-Ficoll. This finding suggests that downregulation of Bcl6 is a consequence of commitment to plasmablast differentiation and not the cause. Importantly, no Bcl6 mRNA expressing CD138$^{+}$ plasmablasts were found to co-express Blimp1. Although Blimp1 and Bcl6 proteins have been shown to down-regulate the expression of each other (Reljic, *et al*, 2000; Tunyaplin, *et al*, 2004; Shaffer, *et al,* 2002) in this situation Bcl-6 mRNA levels fall without the influence of Blimp1. These finding indicate a clear order of maturation during plasmablast formation in the response to 1μg NP-Ficoll. CD138 is first up-regulated; this is followed by down regulation of Bcl-6 mRNA in those cells expressing this message; only after these first two stages are completed is Blimp1 mRNA up-regulated.

### *4.1.4 Probing the relationship of high level IRF4 expression to plasmablast development*

On the basis of immunohistology, flow cytometry and RT2-PCR there is a consistent finding that AFC express high levels of IRF4. This section probes the temporal relationship between the upregulation of IRF4 protein and/or IRF4 mRNA and the transition from B blast to plasmablast. Flow cytometric dot plots of 48 hour NP$^{+}$ cells both from the 1 and 30μg responses shows that in 5 of 9 mice more cells expressed high levels of IRF4 protein than expressed surface CD138 (Fig 4.6A). In the 4 mice where this was not the case the number of AFC as judged by CD138

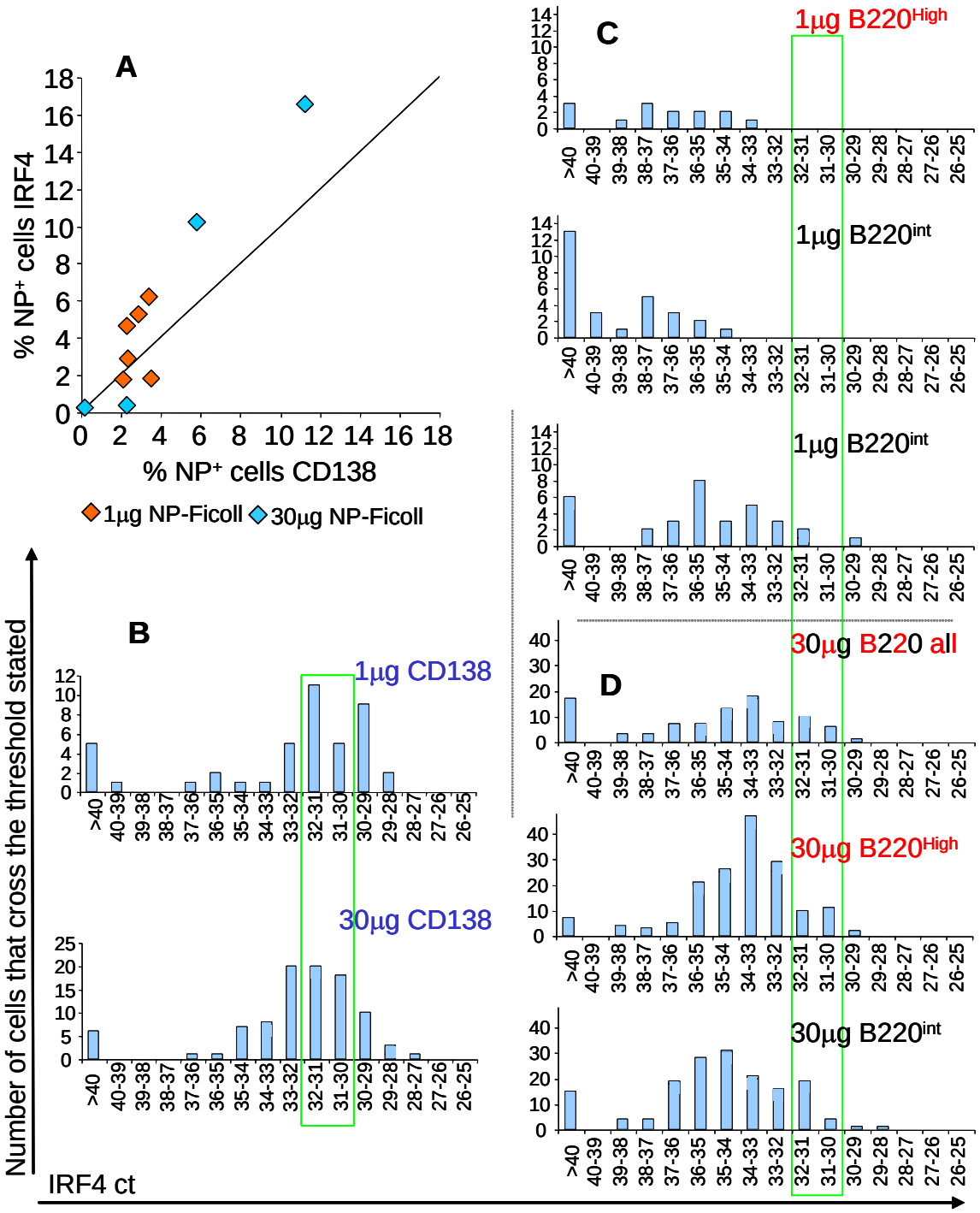


Figure 4.6: Expression of IRF4 precedes CD138 expression at 48 hours

**A**. The percentage of NP+ cells that express CD138 and IRF4 at 48 hours in response to 30µg (blue diamonds) or 1µg (orange diamonds) NP-Ficoll. It would appear that IRF4 is expressed prior to CD138 protein. **B.** CD138+ cells in the response to both 1µg and 30µg NP-Ficoll show high expression of IRF4 mRNA. D. CD138- cells in the response to 30µg NP-Ficoll show a proportion of cells that are in the same range as CD138+ cells (indicated by the green rectangle). C**.** CD138– cells in the response to 1µg NP-Ficoll shows very few of these IRF4high cells. The number of cells that express IRF4 mRNA at the indicated Ct is indicated in each graph.

expression was low. Thus while IRF4 protein is up-regulated from intermediate to high levels before CD138 in many cells this is not always the case. When the number of $CD138^+$ cells is higher than the number of $IRF4^+$ cells might this be due to numerous background plasma cells? This is unlikely; CD138 expression is lost as AFC mature and Ki67 negative plasmablasts, mature plasma cells, remain IRF4 high.

Among $CD138^+$ $NP^+$ cells at 48 hours after either 1 or 30 μg NP-Ficoll there is a broadly normal distribution of IRF4 mRNA expression (Fig 4.6B). This figure boxes the peak of the distribution of IRF4 mRNA levels (green rectangle). As was described in the previous chapter a small proportion of $NP^+$ $CD138^-$ B blasts at 48 hours after 30μg NP-Ficoll have this high level of IRF4 mRNA. When the 1μg response is studied in this way almost no $NP^+$ $CD138^-$ B blasts were found that had this high level of IRF4 mRNA. Taken together these data show that while IFR4 mRNA may be upregulated to high levels in B blasts before they differentiate into plasmablasts this is not always the case. The data seem to support a tentative conclusion that high level IRF4 protein expression is not essential for the production of $CD138^+$, $Blimp1^+$ $Ki67^+$ plasmablasts. On the other hand it seems likely that most if not all cells that differentiate into plasmablasts express at least intermediate levels of IRF4 protein before making the transition to plasmablast.

### *4.1.5. Why is there a reduction in the $B220^{int}$ population in response to 1μg NP-Ficoll?*

Compared to the response to 30μg NP-Ficoll the proportion of $B220^{int}$ cells in the response to 1μg NP-Ficoll is greatly reduced (Fig 4.4). This, coupled with the high Bcl6 mRNA expression observed in this population (Fig 4.5) calls into question

the origins of these cells. There seem two possible explanations for this that are based on whether the $B220^{int}$ cells are derived from cells that were $B220^{high}$ at the time of immunization, or are cells were that were $B220^{int}$ *ab initio*. The second possibility requires there to be a $B220^{int}$ $NP^{+}$ population outside the spleen, for essentially all the $NP^{+}$ cells in the spleen from naïve QMxB6 mice are $B220^{high}$. The most obvious $B200^{int}$ B cell population are B1 cells. In support of this a strong B1b cell response to NP-Ficoll is well documented (Hsu, *et al*, 2004). However, reports of such responses by QM B cells have not been published. The two possibilities suggested are not mutually exclusive and require further study. In either case the relatively small production of $B220^{int}$ B blasts after 1μg NP-Ficoll seems likely to be the consequence of a weaker BCR signal induced by this low dose of antigen.

### *4.1.6. Discussion*

#### 4.1.6.1. What role does antigen signalling play in the development of B blasts to plasmablasts?

Calculations of the number of cells that respond to 1μg NP-Ficoll suggest that two thirds of the NP-binding cells in the spleen participate in the response. However, at 48 hours post immunization a number of the NP-binding cells express levels of B220 and IRF4 protein that are similar to those seen prior to immunization (Fig4.4A and B). Plasmablasts do form but they are much reduced compared to that observed in the 30μg response, indeed, the numbers only slightly increase between 48 and 96 hours (Fig 4.1B). It would appear that the majority of the cells that move and proliferate in response to antigen undergo apoptosis or exit the response. In the response to NP-Ficoll, B cells specific for NP but deficient in btk (a protein kinase downstream of the BCR), proliferate and migrate to the T zone but do not

differentiate into plasmablasts (Vinuesa¸ *et al*, 2001). This deficiency is over come through the use of anti CD40 treatment which provides a secondary signal to stimulate further proliferation and development. This data and the observed effect of the amount of antigen and responding precursors on the formation of GC and plasmablasts in response to NP-Ficoll (Garcia de Vinuesa, *et al*, 2000; Fig 4.1) highlight the importance of a strong BCR signal on B cell differentiation and also the ability of secondary signals, intrinsic or external, to affect the outcome of the response.

The absence of CSR and AID expression in this response may be due to poor signalling from the BCR. Indeed, it has been reported that *in vitro* CSR is an intrinsic event and related to proliferation of the responding cell (Deenick, *et al*, 1999). In the response to 1µg NP-Ficoll the cells proliferate but appear to exit the response and this may indeed be consistent with this finding. However, there have also been a number of studies that emphasise the role of accessory cells and the milieu on CSR in the T independent response (Xu, *et al*, 2008; Litinkskiy, *et al*, 2002; Xu, *et al*, 2007; Gao, *et al*, 2008). These cells act either in response to factors secreted by activated B cells, or by the antigen itself. As such, reduced antigen affects not just the B cells, but also the environment that might otherwise progress the response. This study did not provide separation of both factors but they are not mutually exclusive.

#### 4.1.6.2 What role does IRF4 play in the response to antigen?

Studies of the role of IRF4 in conditional knock out mice, and protein expression during a normal response have indicated that IRF4 is necessary for CSR and the differentiation of B blasts into plasma cells (Klein, *et al*, 2006; Sciammas, *et*

*al*, 2006; Falini, *et al*, 2000). Although IRF4 is certainly necessary for these processes to occur, the response to 1µg NP-Ficoll indicates that expression of IRF4 is not sufficient for either CSR or plasmablast differentiation. Indeed, whilst CSR is associated with B cells that express intermediate levels of IRF4 it clearly not causative as intermediate levels were observed in responding $NP^+$ cells without any substantial AID expression.

Low levels of IRF4 are associated with GC (Falini, *et al*, 2000) and $IRF4^{neg}$ cells are observed within GC in response to 30µg NP-Ficoll (Fig. 3.19). However, the large proportion of $IRF4^{neg}$ cells at 48 hours in the 1µg response, in which few GC are formed and many cells cease to participate, suggests that not all the $IRF4^{neg}$ cells in the 30µg response hours go on to found GC.

4.1.6.3 What is the role and origin of the $B220^{int}$population in the response to NP-Ficoll?

Whilst $B220^{int}$ cells made up around 40% of the responding cells at 48 hours in response to 30µg NP-Ficoll the $B220^{int}$ population is greatly reduced in the response to 1µg (Fig 4.4). Intermediate expression of B220 is associated with memory B cells, B1 cells and B cell differentiation to antigen (Section 3.6.4). In the response to 30µg NP-Ficoll none of these possibilities could be ruled out. The main clue as to the origins of the $B220^{int}$ population perhaps lies in their expression of Bcl6 mRNA. In both the 30µg and 1µg responses the $B220^{high}$ and intermediate populations expressed identical levels of Bcl6 and AID mRNA at 48 hours (Fig. 4.5). It would appear reasonable to suggest that they are behaving in a similar fashion. Is this because $B220^{int}$ cells are derived from $B220^{high}$ precursors and down regulate B220 in a stochastic fashion, or, if the $B220^{int}$ population is derived from B1 cells,

they react to antigen in the same way as the splenic MZ cells, or both? B1 cells are reported to have lower levels of Bcl6 expression and are quicker to express Blimp1 (Fairfax, *et al*, 2007) than B2 cells. Indeed, a peak in Blimp1 expression is seen at 18 hours after administration of NP-Ficoll (Vinuesa, *et al*, 2001). Perhaps, in the absence of a strong antigenic stimulus, the involvement of B1 cells is restricted to form the plasmablasts population observed at 48 hours. Greater availability of antigen and BCR cross linking in the 30μg response allows the response to progress and the involvement of B1 cells, or differentiation of splenic B cells, to occur and expand.

**4.2. If there is a higher proportion of switched AFC at 96 hours is this reflected in a higher proportion of B blasts expressing AID at 48 hours after immunization?**

Class switching was observed in the response to 30μg NP-Ficoll at 48 hours in ~10% of the responding $CD138^-$ cells. As this was co-incident with the detection of switched transcripts to $IgG_3$ it was concluded that it is likely that there was a functional association between the AID expression and CSR. The response to 1μg NP-Ficoll in QMxB6 almost eliminated class switching and very little or no AID expression was observed. To provide further evidence for the role of AID in CSR in the early plasmablast response a model was set up where the proportion of cells responding to NP-Ficoll that undergoing CSR is increased. Polyinosinic:polycytidylic acid (poly I:C) has been shown to act as potentiator of class switching when given at the time of immunization (Le Bon, *et al*, 2001). It was thought that co-immunization with poly I:C and 30μg NP-Ficoll might increase class switching compared to the original response to 30μg NP-Ficoll alone, thereby providing a means to see if a

higher proportion of cells switching is associated with an increased proportion of B blasts expressing AID.

*4.2.1. Co-immunization with poly I:C and NP-Ficoll increases the proportion of switched AFC*

The response of the QMxB6 mice to 30μg NP-Ficoll alone or with poly I:C looked similar at 48 hours after immunization (Fig 4.7, top row). Both have large numbers of $NP^+$ B blasts in the T zone and MZ with a similar number of plasmablasts in the red pulp. At this stage there is no GC formation in either response. At 96 hours after immunization both the response to NP-Ficoll alone and that with poly I:C produced large NP-specific GC and red pulp plasmablasts (Fig 4.7, bottom row). The size of the GC is comparable in the two groups (Fig 4.8A). By contrast the mice given poly I:C with NP-Ficoll had significantly fewer plasmablasts at 96 hours (Fig 4.7 bottom row and 4.8A). T cell independent responses typically result in a crash in plasmablast numbers as the supporting cells in red pulp are overloaded (Garcia de Vinuesa, *et al*, 1999). The decline in plasmablasts at 96 hours might indicate that in the co-immunized response this crash in plasmablast number is advanced compared to the response induced by NP-Ficoll alone.

A comparison of the number of cells switched to $IgG_3$ and $IgG_1$ at 48 and 96 hours shows that co-immunization with poly I:C and NP-Ficoll induces greater CSR than immunization with 30μg NP-Ficoll alone (Fig 4.8B). Of particular interest is that $NP^+$ cells expressing $IgG_3$ protein are observed at 48 hours in the mice given poly I:C with NP-Ficoll. Thus the poly I:C given with NP-Ficoll not only increases the proportion of cells undergoing CSR but induces CSR at an earlier time than when mice are immunized with NP-Ficoll alone.

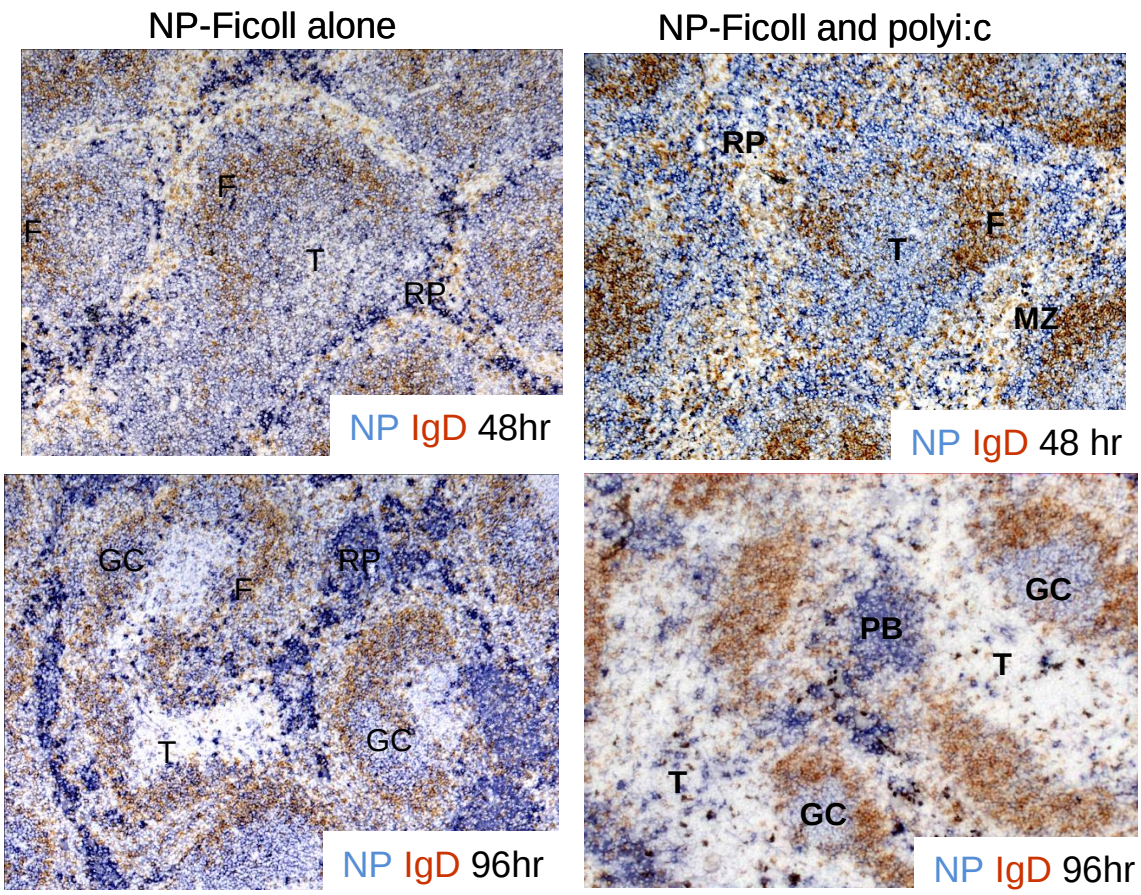


Figure 4.7: Co-immunization with 30μg NP-Ficoll and 10μg poly I:C induces germinal centre, plasmablasts and a larger switched response.

The response to NP-Ficoll with poly I:C (right hand photomicrographs) is similar to that of NP-Ficoll alone (left) at 48 hours with NP binding cells are seen in throughout the T zone (T) and marginal zone with some $NP^+$ cells located among the brown-stained $IgD^+$ B cells in the follicle (F). Plasmablasts are induced in both instances in the red pulp (RP) and at extrafollicular sites but by 96 hours there appear to be fewer NP-specific AFC in the mice given poly I:C with NP-Ficoll. Germinal centres (GC) are present in both groups at 96 hours and appear to be of comparable size. Quantification of the extent of the germinal centre and plasmablast response is shown in the next figure.

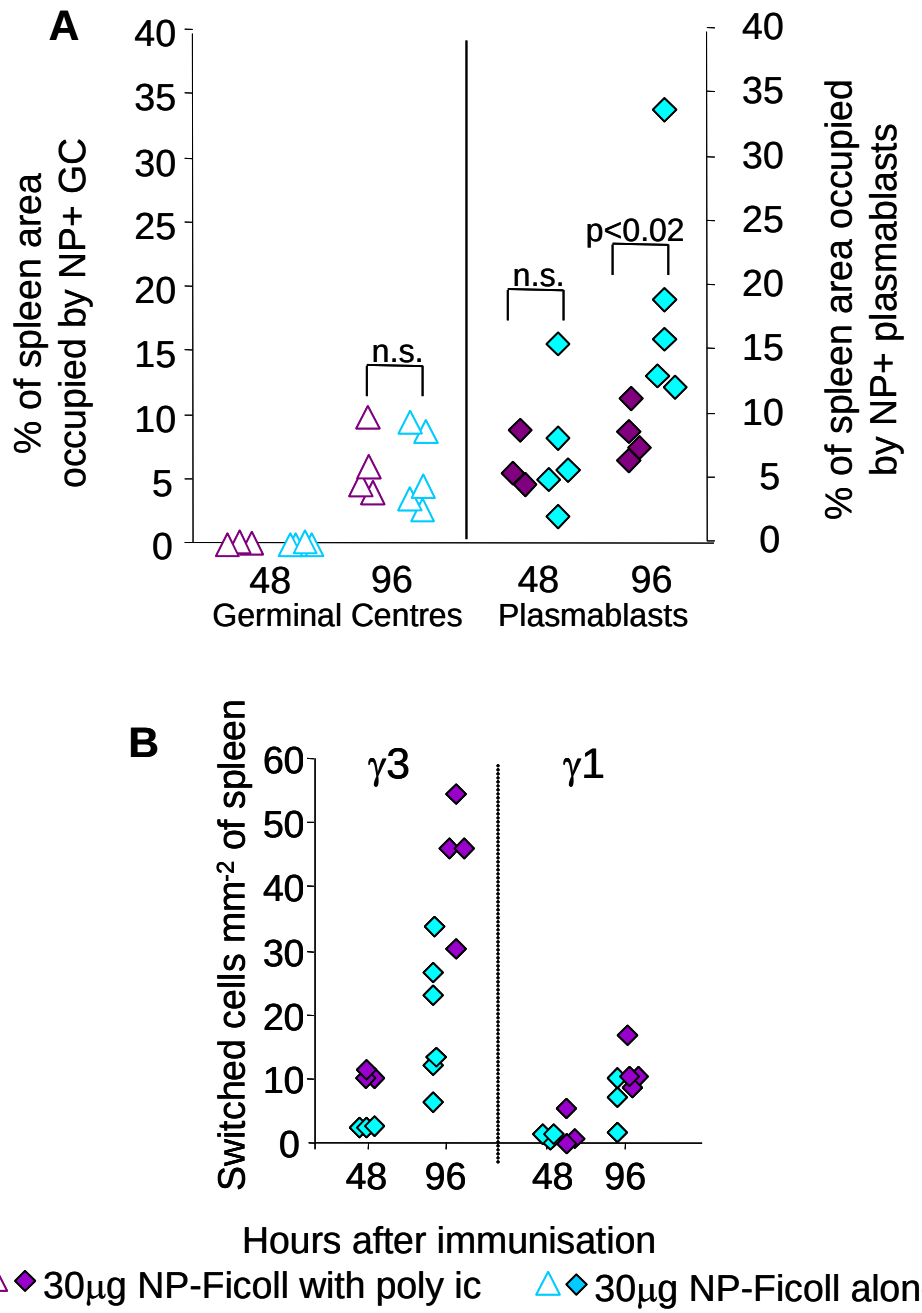


Figure 4.8: The germinal centre and plasmablast response to 30μg NP-Ficoll with polyi:c or alone in QMxB6 mice

**A**. The proportion of spleen area occupied by $NP^+$ germinal centres in the response to 30μg NP-Ficoll with, or without poly I:C is similar at both 48 and 96 hours, while the proportion of the spleen occupied by plasmablasts as assessed by strong cytoplasmic NP binding is reduced in mice given poly I:C with NP-Ficoll. Co-immunization is indicated by purple diamonds, 30μg NP-Ficoll alone by blue diamonds. **B**. When poly I:C is given with NP-Ficoll a higher proportion of cells have switched and switched cells are seen earlier in the response.

4.2.1.1 FACS analysis shows that mice given poly I:C with NP-Ficoll have more plasmablasts at 48 hours than mice given NP-Ficoll alone

Flow cytometric analysis shows that the number of $CD138^+$ $NP^+$ cells 48 hours post immunization is significantly greater when poly I:C is added with 30μg NP-Ficoll (Fig 4.9A, right hand graph). The number of $CD138^-$ $NP^+$ cells induced at 48 hours is similar in the groups of mice. The proportion of $B220^{int}$ $NP^+$ cells at this time is smaller when poly I:C is added with 30μg NP-Ficoll and the proportion of $B220^{high}$ $NP^+$ cells is increased (Fig. 4.9A).

The number of $NP^+$ cells that are $IRF4^{high}$ 48 hours is greater when poly I:C is added with 30μg NP-Ficoll (Fig 4.9B). The increased number of $IRF4^{high}$ and $CD138^+$ cells at 48 hours in the group that received poly I:C indicates the AFC response has been slightly advanced compared to that in the mice given NP-Ficoll only. The increased number of plasmablasts induced by 48 hours when poly I:C is added with NP-Ficoll is consistent with poly I:C causing one or a combination of the following: i) increasing the number of B cells recruited into the response, ii) accelerating the process of B blast differentiation into plasmablasts, iii) increasing the rate of proliferation of the responding cells, iv) reducing apoptosis among the responding cells. That the number of $CD138^+$ cells at 48 hours is the same in response to both NP-Ficoll alone and with poly I:C suggests that this increase in proliferation was not universal. In addition, no acceleration of GC response was seen. Poly I:C is a know inducer of type 1 interferons and these have been shown to reduce apoptosis of CD4 T cells in inflammatory responses (Scheel-Toellner, *et al*, 1999).

The FACS data also show another key difference between the co-immunized and NP-Ficoll alone response. As in the response to 1μg NP-Ficoll (section 4.1.5)

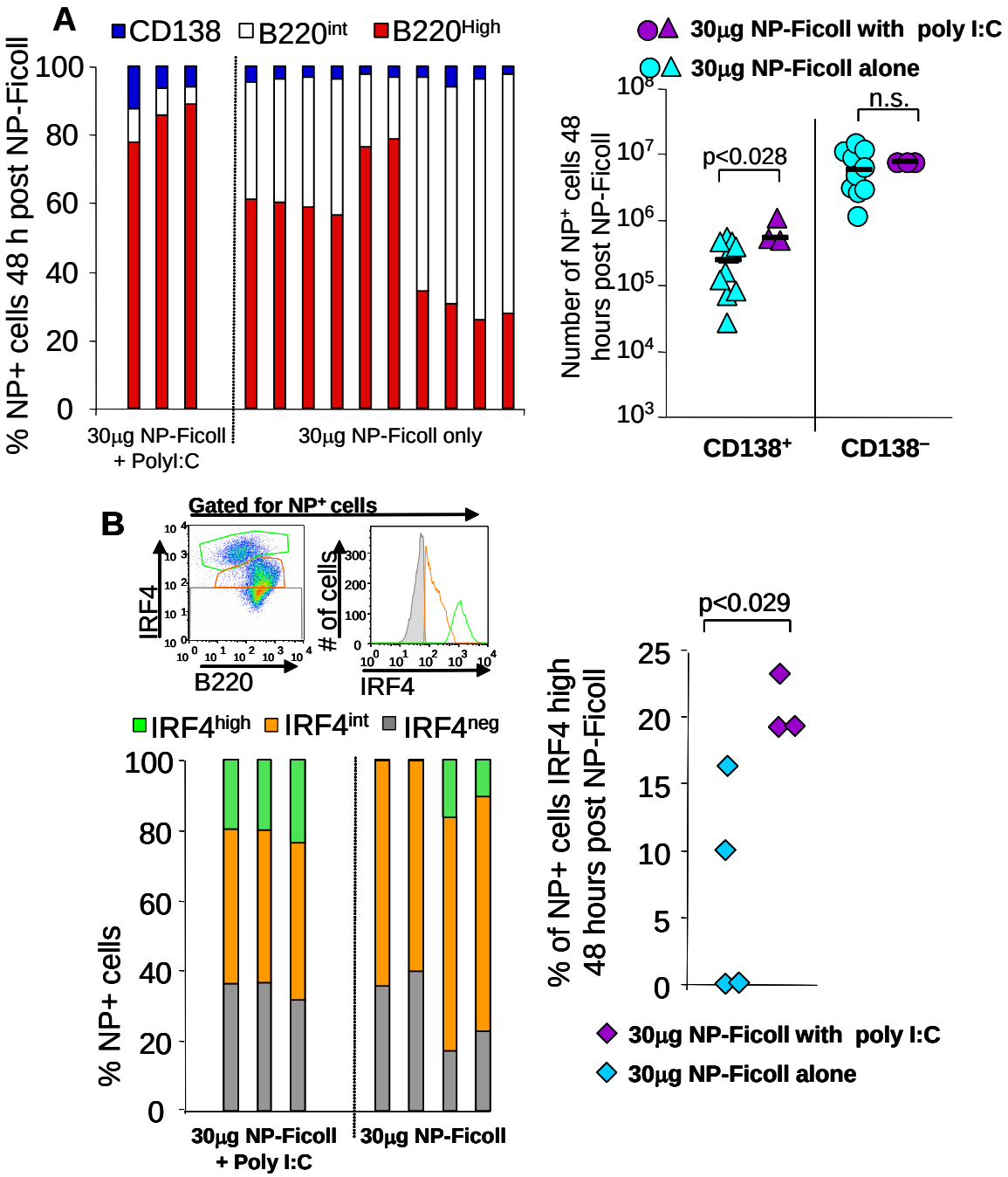


Figure 4.9: The co-immunized response has an advanced plasmablast response compared to 30µg NP-Ficoll alone.

**A.** The proportions of NP responding cells that are CD138+, B220high CD138- and B220int CD138- in the response to 30µg NP-Ficoll and 30µg NP-Ficoll with poly I:C. The addition of poly I:C results in the production of a significantly greater number of NP+, CD138+ cells at 48 hours although the number of NP+ CD138– cells is similar. **B.** The effect of co-immunization of poly I:C with NP-Ficoll to the expression of IRF4 by NP+ cells 48 hours post immunizations. The FACS dot plots and histogram show data for a mouse that had received poly I:C with NP-Ficoll. The gates used to quantify high, intermediate and low IRF4 expression are shown in the FACS dot plots. There are significantly more IRF4Hi NP+ cells in the poly I:C group at 48 hours.

there is a dramatically reduced $B220^{int}$ population at 48 hours (Fig 4.10A). Further studies are required to determine if the co-immunized response reduces or prevents the recruitment of $B220^{int}$ B1b cells, or reduces the differentiation of $B220^{high}$ cells recruited into the response into $B220^{int}$, $CD138^{-}$ cells. In this response with high dose NP-Ficoll it is unlikely that there is a significant number of non-responding $NP^{+}$ cells. These points are discussed further in section 4.3.2.

There appears to be a reduction in the number of cells that express intermediate levels of IRF4 at 48 hours when poly I:C is given with NP-Ficoll (Fig 4.9B). This may be due to the early differentiation $IRF4^{int}$ cells into $IRF4^{high}$ cells, for the total proportion of cells expressing IRF4 protein is not significantly different between the two groups. The similar proportion of $IRF4^{low/neg}$ cells in both groups at 48 hours is consistent with the concept that this population of B blasts in responses to high-dose 30μg NP-Ficoll are destined to differentiate into GC cells; for as is shown in Fig 4.8A the GC response is of similar magnitude in the two groups.

*4.2.2. Co-immunization increases the amount of switching without an increase in AID expression at 48 hours*

As described above and shown in Fig 4.8 poly I:C added with NP-Ficoll resulted in a doubling of the median number of AFC that were producing $IgG_3$ at 96 hours (Inset, Fig 4.10). It was therefore surprising to find that the proportion of B blasts expressing AID at 48 hours, as assessed by RT2-PCR, was much smaller when poly I:C had been added to NP-Ficoll (Fig 4.10). Thus in the poly I:C group of 280 B blasts were 12 expressed AID, while in the group receiving NP-Ficoll only of 700 B blasts analyzed 83 were $AID^{+}$; this difference is significant $p<0.0003$ (two sided Chi square test).

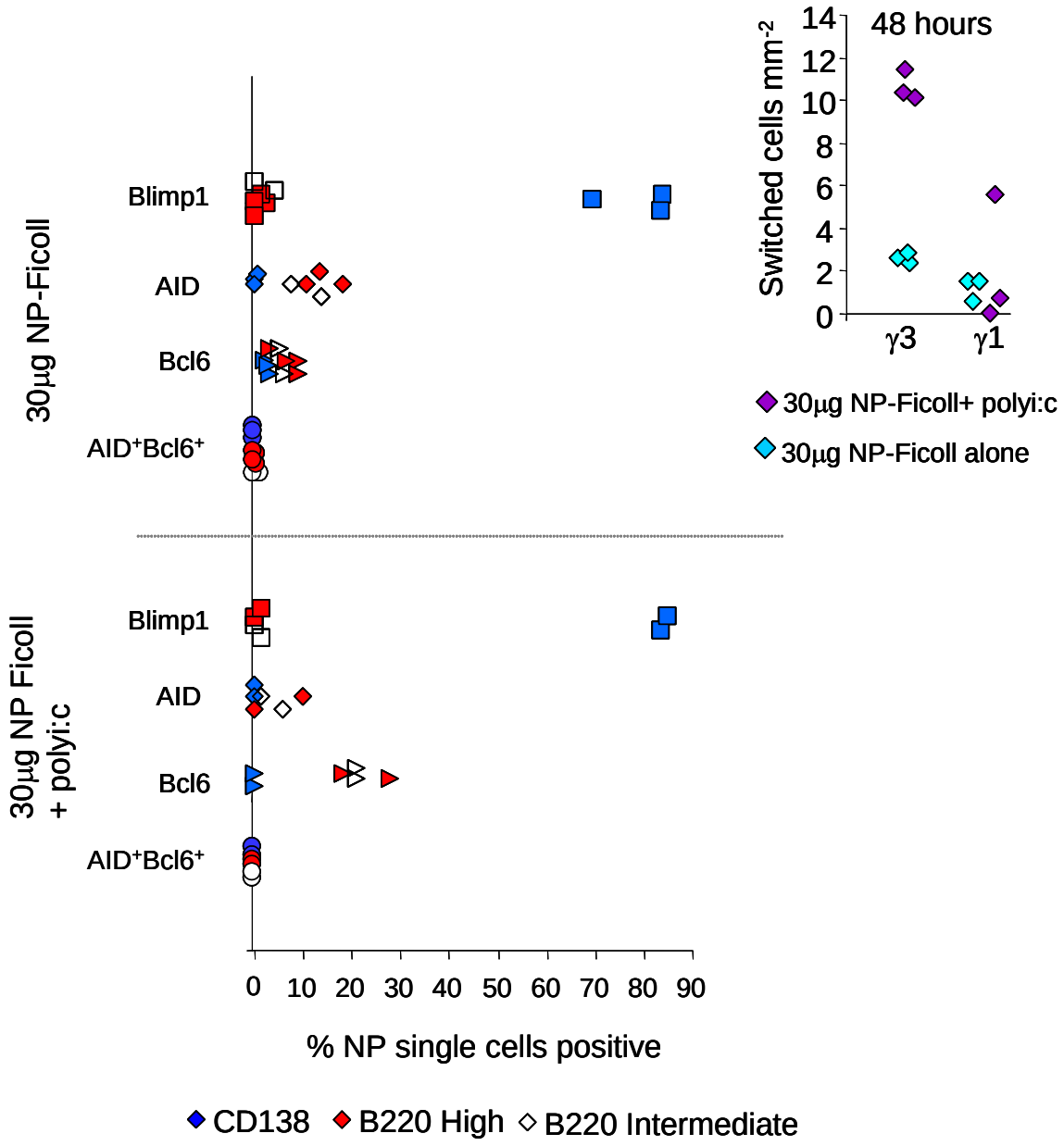


Figure 4.10: Single cell RT2-PCR on NP-Ficoll and polyI:C co-immunized NP$^+$ cells at 48 hours shows lower AID expression than that seen in response to 30μgNP-Ficoll alone.

The expression of Blimp1 and AID$^+$ with BCl6$^+$ is similar in the co-immunized and NP-Ficoll responses whilst there is a greater proportion of Bcl6 expression in co-immunized cells and less AID expression. The percentage of cells expressing the genes either singly or together as shown. CD138$^+$ cells are blue diamonds, B220$^{high}$ cells red diamonds, B220$^{int}$ cells are white diamonds. Inset shows switching at 48 hours in both responses.

*4.2.3. Expression of Bcl6 at 48 hours is greater than that observed in the response to NP-Ficoll alone*

The response of the QMxB6 mouse to 1μg and 30μg NP-Ficoll showed that Bcl6 mRNA expression does not exclude cells from the AFC pathway, and is therefore not a certain marker of GC cell commitment. This experiment was not expected to address the role of Bcl6 in the B cell response to antigen, however, a higher level of Bcl6 expression was seen in the $CD138^-$ cells 48 hours post immunization than in the response to 30μg NP-Ficoll (Fig 4.10). This proportion is coincident with the slightly increased $IRF4^{low/neg}$ population seen at this time (Fig 4.9) although that may be co-incidental. The increased proportion of $Bcl6^+$ cells is not associated with the induction of larger GC in the mice immunized with NP-Ficoll plus poly I:C, making it unlikely that the excess $Bcl6^+$ cells were destined to becoming GC cells. GC formation was not accelerated as no $NP^+$ cells co-expressing AID and Bcl6 mRNA were seen at 48 hours (Fig 4.10). As 30μg NP-Ficoll were given it is unlikely that the increased proportion of $Bcl6^+$ cells among $NP^+$ cells at 48 hours is attributable to non-responding cells. The role of Bcl6 mRNA in all three models is discussed in Section 4.3.2.

*4.2.4 None of the CD138 population in response to co-immunization expresses Bcl6*

As in the other responses studied, the response to poly I:C with 30μg NP-Ficoll induced Blimp1 expression solely in $CD138^+$ cells. In the 30μg and 1μg NP-Ficoll responses without added poly I:C a small proportion of $Bcl6^+$ cells were seen at 48 hours in the small $NP^+$ $CD138^+$ population in some mice (Fig 4.5.A). By contrast, neither of the mice given co-immunized with poly I:C and NP-Ficoll

analyzed by single cell RT2-PCR had any Bcl-$6^+$ cells in the $CD138^+$ fraction. If, as suggested by earlier data (Section 3.6.2), CD138 is upregulated prior to expression of Blimp1 and downregulation of Bcl6, this furthers the suggestion co-immunization advances the plasmablast response.

## 4.3 Discussion

### *4.3.2. The effect of TLR co-stimulation on class switch recombination and plasmablast proliferation.*

Co-immunized QMxB6 mice showed a population of $IgG_3$ positive cells at 48 hours prior to their appearance in response to NP-Ficoll alone. In addition, there were greater numbers of both $IgG_3$ and $IgG_1$ cells in the co-immunized response at 96 hours (Fig. 4.9A). For CSR to have occurred at 48 hours AID must have been expressed during the first day, unless the switching in this situation followed a novel and highly iconoclastic AID-independent switching process. In future experiments it will be necessary to look for AID expression during the second day of this response. AID independent ssDNA break events that have been reported (Arudchandran, *et al*, 2008). Perhaps these are responsible for the low expression of AID mRNA. Although this does provide an alternative, or complimentary, means to achieve CSR the most prudent experiment would be the investigation of earlier time points during the co-immunized response.

There was an increased proportion and number of plasmablasts at 48 hours in the co-immunized response compared to that induced by NP-Ficoll alone without an obvious overall increase in the number of responding cells (Fig 4.10). This favours earlier differentiation to plasmablasts or a higher proportion of cells

committing to becoming plasmablasts or a combination of these. Theoretically there might also have been selective increased proliferation in the plasmablast precursors. These possibilities could be investigated further, but this was not considered a priority in the present programme. This especially applied as the results with the 1μg NP-Ficoll response show that AID is not expressed in 48 hour B blasts in a response that is not associated with switching.

The role of poly I:C has on plasmablasts and proliferation of B blasts has been the subject of a number of studies. Poly I:C binds TLR3 (Alexopoulou, *et al*, 2001) and in doing so increases α-IFN secretion by macrophages and splenocytes (Field, *et al*, 1967; Gisler, *et al*, 1974; Alexopoulou, *et al*, 2001, Le Bon, *et al*, 2001). In a similar fashion human tonsil B cells stimulated with poly I:C and IL-10 undergo proliferation and are induced to express AID in a T cell independent manner. This is accentuated by BAFF, which is released by DC after poly I:C exposure (Xu, *et al*, 2008). In the present study the effect of poly I:C on the spleen may have increase the external signals provided by the milieu as well as increasing the cell autonomous switch signals. Alternatively proliferation in switched plasmablast precursors may provide the increased numbers. Perhaps BAFF, important for plasmablast survival (Balazs, *et al*, 2002), is induced by poly I:C and increases retention of switched plasmablasts. Both suggestions could be validated through RT2-PCR at earlier time points in the response, with the effect of poly I:C on proliferation (through CFSE studies) or quantification of BAFFR and BAFF expression paid particular attention.

*4.3.3. What role does Bcl6 play in the co-immunized response?*

In comparison to the 30μg NP-Ficoll response both the 1μg and co-immunized responses had high levels of Bcl6 at 48 hours (Fig. 4.5 and 4.11). In the

co-immunization response, the finding of an increased proportion of cells expressing Bcl6 mRNA in $CD138^-$ cells was not anticipated and is not easily explained. This may well be due to delay in the down-regulation of Bcl6 mRNA expressed by about half the splenic B cells in QMxB6 mice, rather than de-novo expression of the mRNA. Cells expressing Bcl6 protein could not be identified using immunohistochemistry at this stage, indicating that this mRNA expressed in the 48 hour B blasts, as in pre-immunization B cells is not being translated. The receptor for poly I:C, TLR3, is present on B1and MZ B cells at high levels and lower levels on recirculating B cells (Barr, *et al*, 2007). As the B cells responding to NP-Ficoll with poly I:C are likely to be B1 and MZ B cells it seems reasonable to conclude that any signaling through TLR3 is contributing to the delay in down-regulation of Bcl-6. Fairfax, *et al*, (2007) reported that peritoneal B1 and splenic MZ cells constitutively express less Bcl6 mRNA than splenic B2 cells. At least a proportion of the $NP^+$ cells in the spleen of QMxB6 mice must express Bcl6 mRNA, as there are more $NP^+$ cells with Bcl6 mRNA than there are $NP^+$ recirculating B cells in these mice.

### *4.3.4. The low proportion of $B200^{int}$ $NP^+$ B blasts at 48 hours in the combined response*

The markedly low proportion of $B220^{int}$ cells in the response to 30μg NP-Ficoll with poly I:C compared to the high proportion of these cells induced with 30μg NP-Ficoll alone is intriguing and deserves further study. Does this reflect a negative effect of poly I:C on the recruitment of B1b cells from the peritoneal cavity into the splenic response? Indirect evidence against the $B220^{int}$ population being B1b cells has already been discussed in Chapter 3. It might be possible to study the role of B1b cells by passive transfer of genetically-tagged NP-specific B1b cells into QMxB6

mice and looking to see if there is differential recruitment in the response to NP-Ficoll alone compared to that with poly I:C.

# Chapter 5

# B blasts in a germinal centre-biased response

## 5.1 The development of a germinal centre-biased B cell response model

The aim of this chapter has been to study the origin, locations and phenotypes of B blasts that are destined to differentiate into germinal centre (GC) B cells. The model used in these experiments was developed in the following way: large GC are formed in rodents primed with alum-precipitated carrier protein given with killed *B. pertussis* and subsequently challenged with soluble hapenated carrier (Liu, *et al*, 1991; Toellner, *et al*, 1996). These GC form in a synchronized manner, are hapten-specific, and reach peak size by 96 hours after immunization. These predictable features of the response have made this an ideal system to analyze the kinetics of GC formation (Liu, *et al*, 1991; Toellner, *et al*, 1996). In wild-type mice this carrier primed response also produces a large synchronized and predictable extrafollicular antibody response (Toellner, *et al*, 1996; Sze, *et al*, 2000). Serendipitously, pilot experiments found that if Quasi Monoclonal (QM) B cells are transferred into carrier primed mice that are then immunized with soluble hapten protein the response is highly biased toward GC formation and extrafollicular responses are only small and erratic. This protocol provides a model to study a response where most of the B blasts are destined to form GC.

The GC-biased response was an adaptation of the carrier primed response model was used (Toellner, *et al,* 2002). Wild type mice are immunised with alum-precipitated chicken gamma globulin (CGG) with killed *B. pertussis* and left for 5 weeks. Congenic QM B cells, which are >60% NP-specific (Cascallo, *et al*, 1996),

are transferred into these mice and 24 hours later they are challenged with *intra peritoneal* (*i.p.*) soluble NP-CGG.

The carrier primed, GC biased T cell dependent (TD) response allowed the following questions to be addressed:

i. As a high proportion of the NP-specific B blasts become GC, how does this affect the phenotype of B blasts 48 hours after challenge with hapten carrier?

ii. Does the GC bias affect the proportion of cells that express low and high levels of IRF4 protein?

iii. Is AID mRNA expressed in the absence of Bcl6 mRNA in a response that is TD and if so does this relate to the proportion of GC with switched NP-specific B cells?

iv. Does reducing the number of responding cells affect the outcome of the response?

The QMxB6 response to 30µg NP-Ficoll showed that $NP^+$ B cells migrate to the T zone shortly after immunization and then proliferate to fill the T zone and overspill into the marginal zone (Section 3.1.2). By 72 hours the NP-binding cells have also formed GC and plasmablasts are present in the red pulp (Section 3.1.3). This model does not provide a reliable control for the carrier primed response as the number of responding cells is much lower when QM cells are transferred into primed C57Bl6 mice. Consequently some carrier primed mice that had received a transfer of

QM B cells were immunized with 30μg NP-Ficoll. This also allows assessment of how the response to NP-Ficoll is affected by having a markedly smaller number of responding B cells.

**5.1 The carrier primed response to NP-CGG is biased towards germinal centres with few plasmablasts.**

As described above C57Bl6 mice were primed with 30μg alum-precipitated CGG with $5x10^8$ heat-killed *B. pertussis i.p.* Five weeks later the mice received between $5x10^5$ and $2x10^6$ NP-specific cells contained in QM x QM splenocyte suspensions. The affect of number of cells transferred is discussed in section 5.7.2. These chimeras were then challenged *i.p.* with 50μg soluble NP-CGG. Seventy two hours after this challenge immunohistochemistry on the chimeras' spleens shows NP-binding cells have begun to enter GC (Fig. 5.1A and B). In most GC the NP-specific cells are located around the edge of preformed GC, but some GC are already filled with NP-specific cells. The GC at this stage contain few if any CGG-specific cells (Fig 5.1C & 5.2C). In addition to the NP-specific blasts in GC there are still many NP binding cells located in the follicular mantle (Figure 5.1A). These follicular mantle $NP^+$ cells are $Ki67^+$ (Fig 5.1B) but most do not express Bcl6 protein. This contrasts with the cells in the GC, which, as expected, express Bcl6 (Fig 5.1C). The size of the $NP^+$ GC at 72 hours are quantified in Fig 5.2 A. In 4 of 6 mice there were some $NP^+$ plasmablasts located in extrafollicular foci at the boundary of the T zone and the red pulp; the remaining 2 mice had few if any $NP^+$ plasmablasts. The relative paucity of $NP^+$ plasmablasts compared with $NP^+$ GC is quantified in Fig. 5.2 A&B. Whilst the carrier primed $NP^+$ GC cover a fifth of the area of those produced during the QMxB6 response to NP-Ficoll (Fig. 5.2B) the number of participating $NP^+$

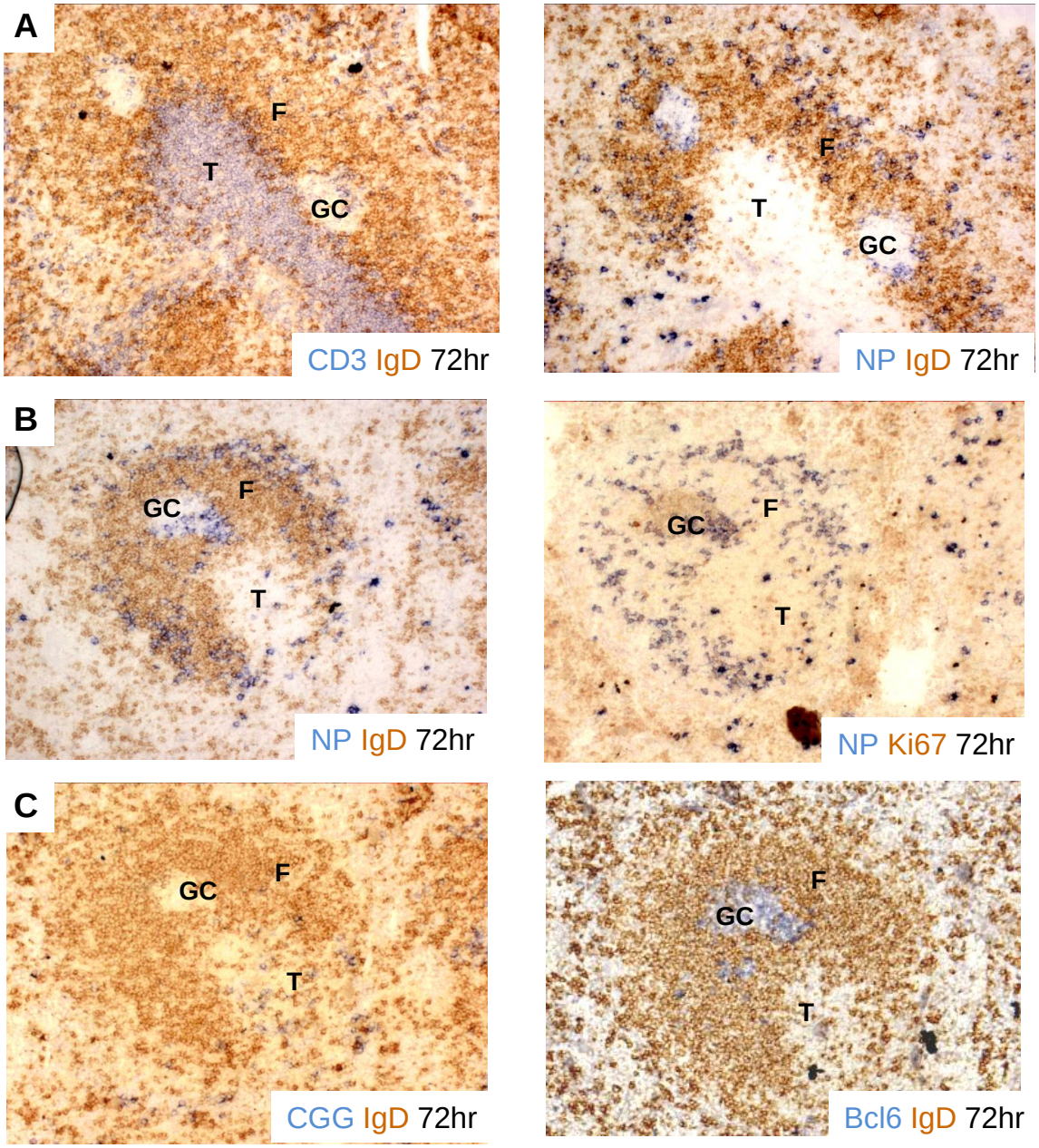


Figure 5.1: The carrier primed response to NP-CGG is biased towards NP$^+$ germinal centres.

**A** and **B**. 72 hours after immunization with NP-CGG, NP binding cells are found in germinal centres (GC). However, NP binding cells are still found in the outer half of the follicle (F) and some appear to have entered the marginal zone. **C**. The response to CGG is characterised by extrafollicular foci of plasmablasts. Staining as described. T= T zone.

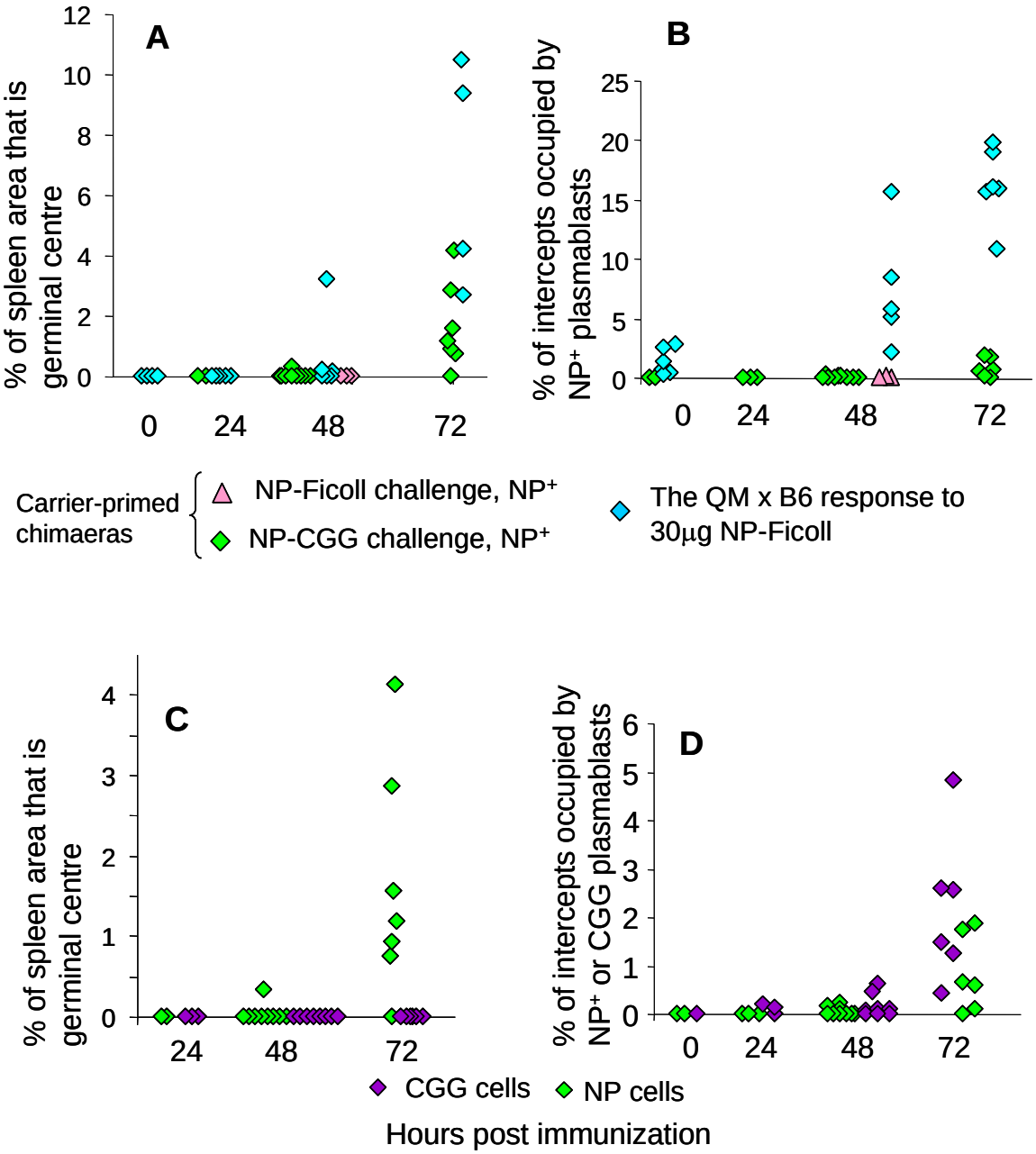


Figure 5.2: The NP+ carrier primed response to NP-CGG is predominantly germinal centre biased although some early plasmablasts are induced.

**A**. NP binding cells (green diamonds) appear in germinal centres on the third day after challenge with NP-CGG. **B**. The proportion of plasmablasts induced by the response to NP-CGG is small compared to the 30μg NP-Ficoll response in QMxB6 mice. **C**. The B cell response to CCG produces no germinal centres. D. The response to CGG is plasmablast based.

cells at 72 hours is 50 times smaller than the number of $NP^+$ cells in the QMxB6 response to 30µg NP-Ficoll at the same time (data not shown). By contrast, the memory CGG-specific response to NP-CGG is associated with the formation of extrafollicular plasmablasts (Fig. 5.2D) without GC formation (Fig. 5.2 C). This is very similar to the CGG response to NP-CGG in carrier primed C57Bl6 mice that have not received QM B cells (Toellner, *et al*, 1996).

## 5.2 NP-CGG induces NP-specific B cells to migrate to follicles and associate with follicular T cells in CGG-primed mice that have received QM B cells

Twenty four hours after immunization with NP-CGG the responding NP-binding cells were characteristically seen clustered at the corners of the follicles nearest the area where the T zone abuts the red pulp (Fig 5.3). In addition, some were dotted within the follicle. Only occasional NP-binding cells were seen at the boundary between the base of the follicle and the T zone. The significance of the clustering of responding B cells at the corner of follicles was investigated by looking to see if these cells were associated with follicular T cells. In naïve mice there are very few follicular T cells, but a subset of responding CD4 T cells start to colonise follicles 72 hours after primary immunization and within 24 hours of secondary immunization (Gulbranson-Judge and MacLennan, 1996; Serre, *et al*, 2009). Figure 5.4A identifies a cluster of NP-binding cells located among $IgD^+$ follicular B cells 24 hours after primed mice had been challenged with NP-CGG. Most of these NP-binding cells are Ki67 positive. It can be seen that some, but not all of NP-binding cells are close to, or directly in contact with, CD3 positive cells (Fig 5.4B). There appears to be follicular commitment of these responding NP-binding cells for they have colonized the whole of the outer proportion of the follicles by 48 hours (Fig

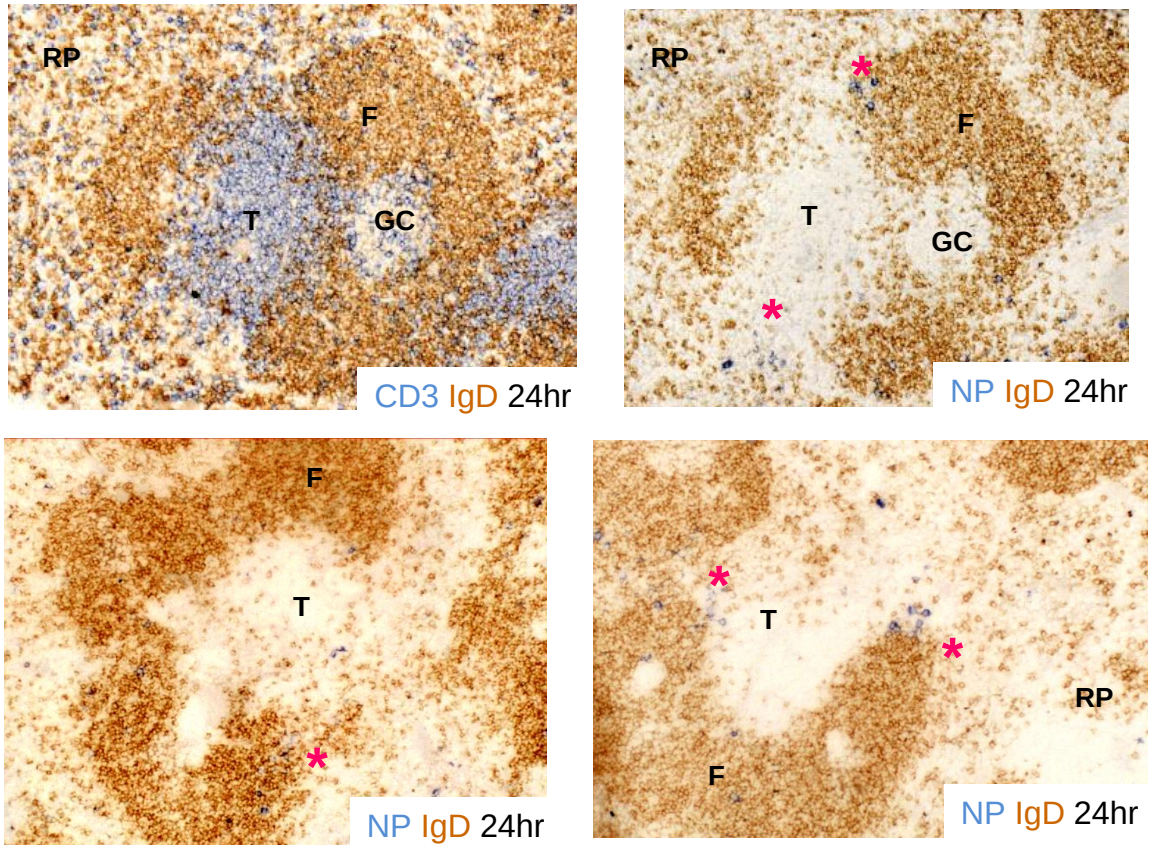


Figure 5.3: NP binding cells appear at the edge of the follicle at the border with the T zone 24 hours after immunization with NP-CGG.

Whilst some $NP^+$ cells are seen in the follicle (F), there is a trend for NP binding cells to be located at the end of the follicle at the border of the T zone (T) and the red pulp (RP). These are indicated by an asterix (*). GC= background germinal centre. Staining as indicated.

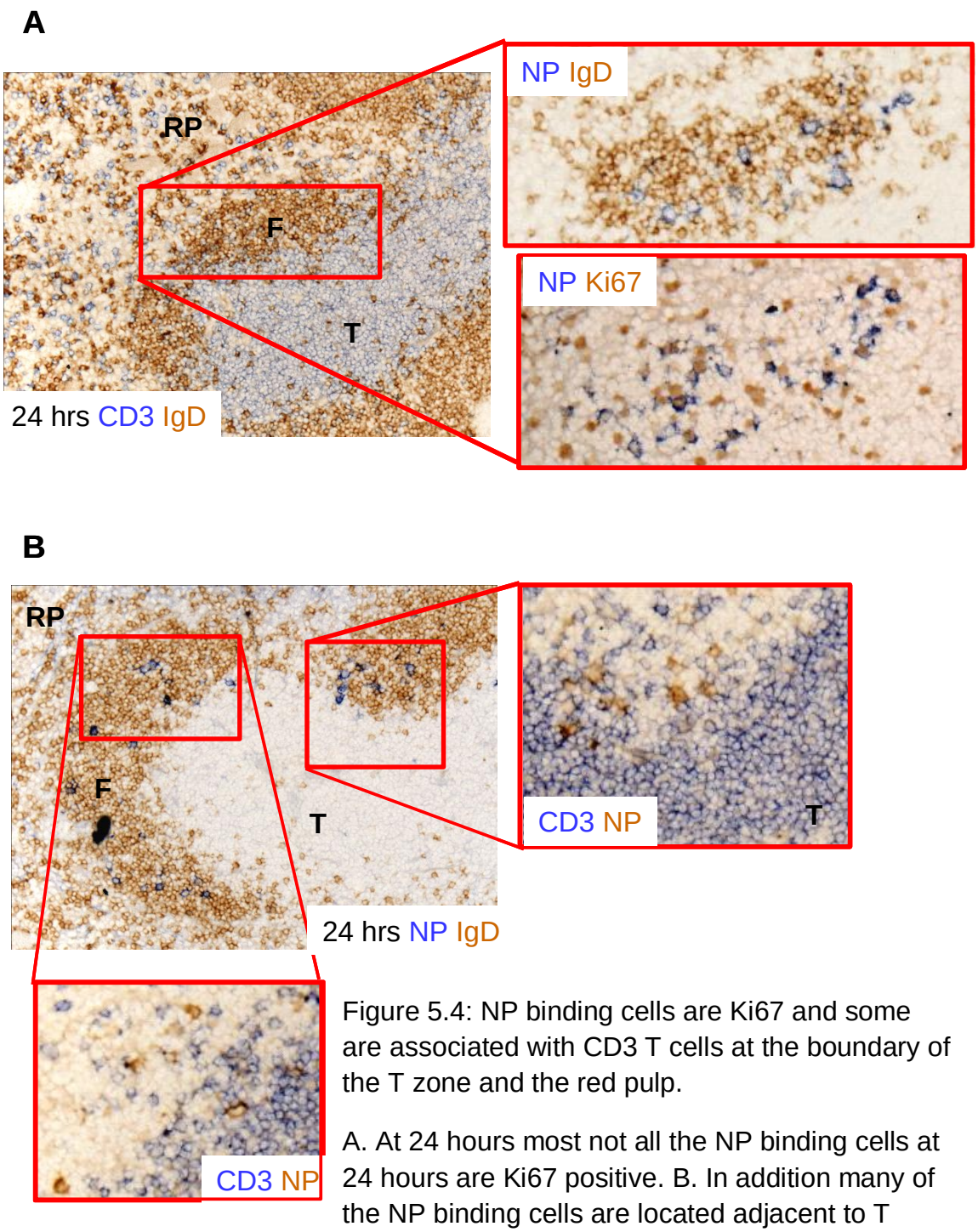


Figure 5.4: NP binding cells are Ki67 and some are associated with CD3 T cells at the boundary of the T zone and the red pulp.

A. At 24 hours most not all the NP binding cells at 24 hours are Ki67 positive. B. In addition many of the NP binding cells are located adjacent to T cells. T = T zone, F = follicle, RP = red pulp, staining as indicated.

5.5A). At this stage the majority (median 71% range 59-81%) are in contact with follicular T cells (Fig 5.5C). As these studies used a two dimensional histological slide it could be assumed that some of the non-associated cells may make contact with T cells above or below the plane observed and so this may be an underestimate. The follicular distribution of the responding $NP^+$ B cells contrasts strikingly with that observed in QMxB6 response to NP-Ficoll, where the responding B cells translocate to the border between the base of follicles and the T zone (described in chapter 3). This difference still applies when the response of carrier primed chimeras to NP-Ficoll was studied (Fig. 5.5B). There is a highly significant difference between the location of NP-binding cells 48 hours after immunizing these chimeras with NP-Ficoll compared with NP-CGG (Fig 5.5D).

This co-location of B blasts and T cells in follicles early in the response of carrier primed chimeras to NP-CGG does not appear to have been described previously. It was not noted in the responses of carrier primed C57Bl6 to NP-CGG (Toellner, *et al*, 1996). This discrepancy may be in part because precursor B cell numbers have been increased by QM B cell transfer and in part because the response is heavily biased to GC formation. This will be considered further in the discussion.

### 5.3. Cell activation and differentiation as assessed by flow cytometry

Flow cytometry of the transferred QM B cells responding to NP-CGG in CGG primed recipients shows that a small CD138 population has emerged by 48 hours. Strikingly this has hardly increased by 72 hours (Fig 5.6A). This is confirmed by the immunohistology on the spleens of chimeras at 72 hours after receiving NP-CGG; 4 mice showed small extra follicular foci of NP-binding plasmablasts and 2 had almost

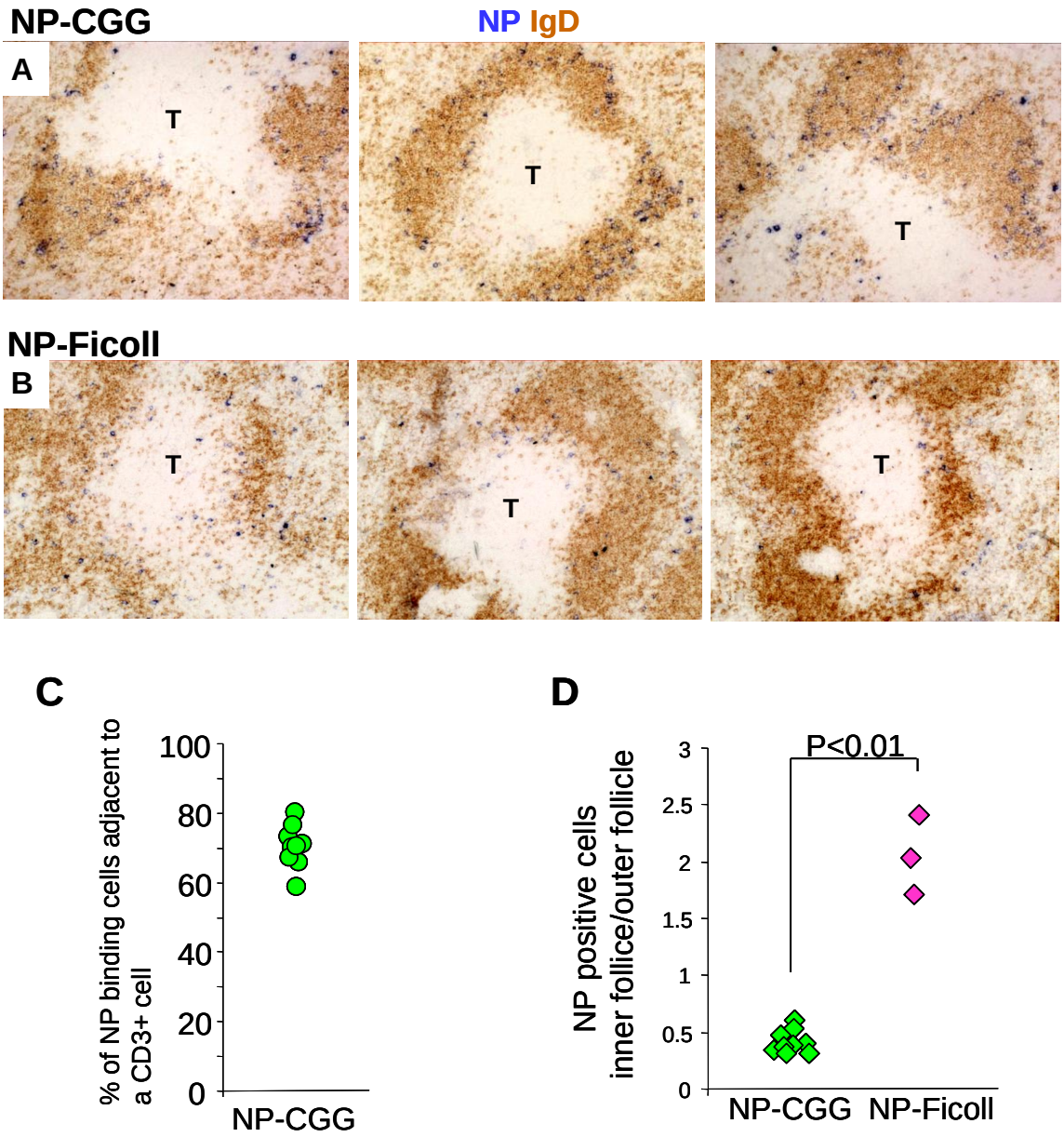


Figure 5.5: The location of NP-binding cells in the follicle at 48 hours during the carrier primed response 50μg NP-CGG or 30μg NP-Ficoll.

**A**. The carrier primed response at 48 hours after immunization with NP-CGG shows that $NP^+$ cells are located towards the outer follicle. **B**. After NP-ficoll administration $NP^+$ cells are located at the T zone follicular boundary. NP stained in blue and IgD, illustrating the follicle, in brown. T= T zone, F= follicle. **C**. The difference between the locations was determined as described in methods (2.13). The statistical difference between the groups was tested using the Mann-Whitney U test result: P<0.01, n1 = 9 and n2= 3. **D**. Most NP binding cells are adjacent to $CD3^+$ cells regardless of their location in respect to the follicle.

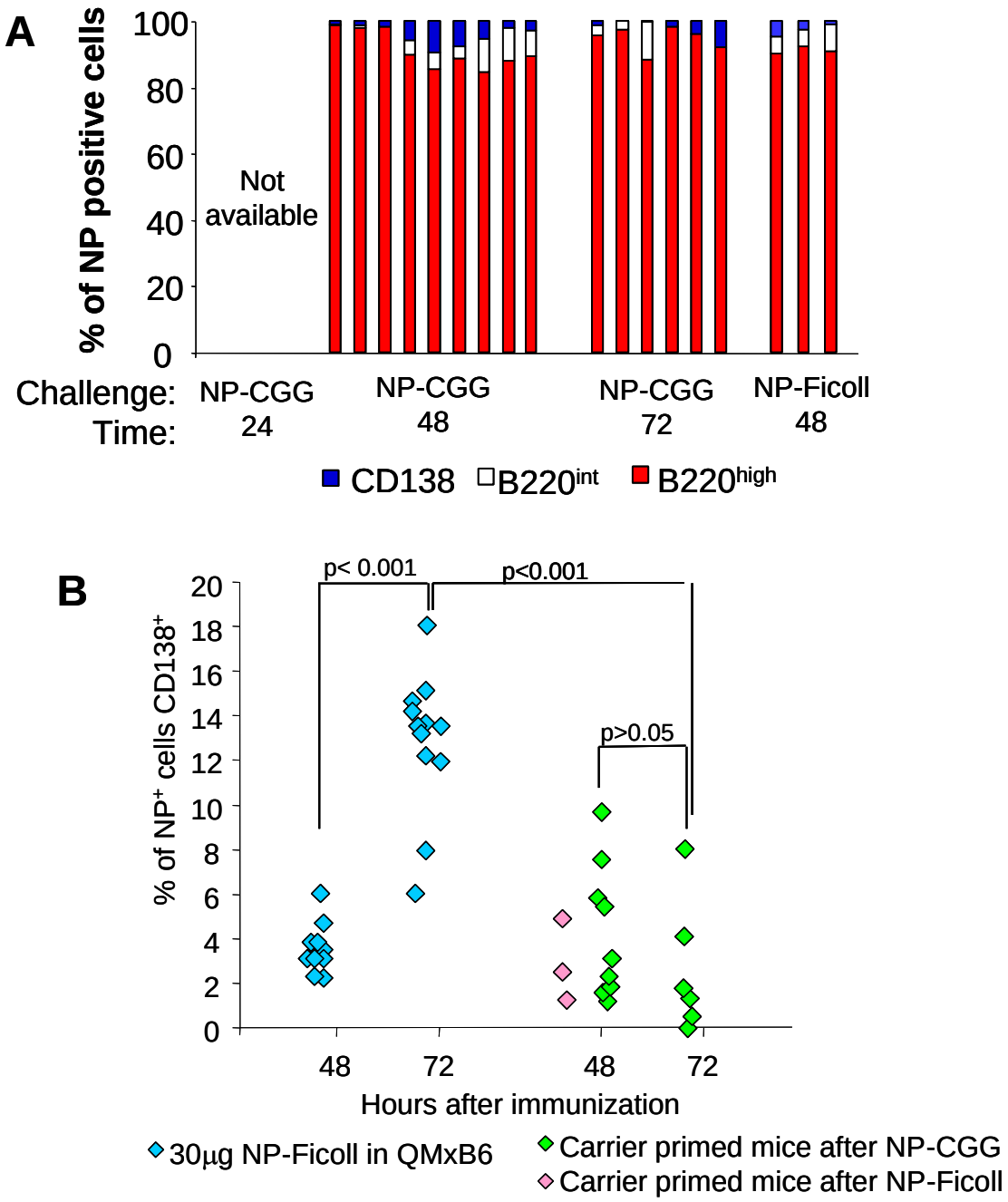


Figure 5.6: The proportion of B220 and CD138+ subsets in NP binding cells reflects the small number of plasmablasts and large numbers of germinal centres produced by the response.

**A.** The proportion of B220high , B220int and CD138+ populations amongst the NP binding cells shows very few CD138+ or B220int cells. **B**. The percentage of NP+ cells that are CD138+ at 48 and 72 hours after immunization with 30μg NP-Ficoll in QMxB6 mice (blue diamonds) and NP-CGG or NP-Ficoll in carrier primed mice that have received QM cells (green and pink diamonds). Whilst there is a large increase in the proportion of CD138+ cells in the QMxB6 response to NP-Ficoll there is no increase in the proportion of CD138+ cells observed in the NP-CGG carrier primed response.

none of these cells. By contrast, all 6 of these 72 hour chimeras had developed $NP^+$ GC. The failure to increase the number of plasmablasts between 48 and 72 hours contrasts with the increase in the proportion of plasmablasts seen between 48 and 72 hours in QM x B6 mice immunized with 30μg NP-Ficoll (Fig 5.6B). On the other hand it resembles the response of QM x B6 mice to 1μg NP-Ficoll (Chapter 4). This finding was considered compatible with the conclusion that many of the responding B blasts underwent apoptosis on the third day after immunization, without differentiating into plasmablasts or GC B cells. In 6 of the 9 mice assessed at 48 hours in the carrier primed response of chimeras to NP-CGG a high proportion of the NP-specific cells were $IRF4^{low/neg}$ (Fig. 5.7A). As a median of 90% of the NP-specific blasts at 48 hours were $Ki67^+$ it might be assumed that most of these cells were participants in the response despite their low IRF4 expression. This also accords with the low dose response to NP-Ficoll in QMxB6 mice, however, that may be co-incidental. The three chimeric mice responding to NP-CGG that had a much higher proportion of the B blasts at 48 hours that were $IRF4^{int}$ might reflect a subgroup where, if these mice had survived, a higher proportion of B blasts would have differentiated to plasmablasts. As described above 4 of 6 mice analyzed at 72 hours had extrafollicular foci of NP-specific plasmablasts, while 2 had none. Low or no expression of IRF4 is associated with GC B cells (Falini, *et al* 2000). It might be that a proportion of the $IRF4^{low/neg}$ cells at 48 hours are GC B cell precursors, but this is not the case for the 48 hour $IRF4^{low/neg}$ B blasts in the 1μg NP-Ficoll response, which induced negligible GC. Because it is not possible to predict the proportion of B blasts analyzed at 48 hours that would have gone on to die, become plasmablasts or form GC, the available data do not allow one to conclude that the level of IRF4 expression of relates to the B blasts' subsequent fate.

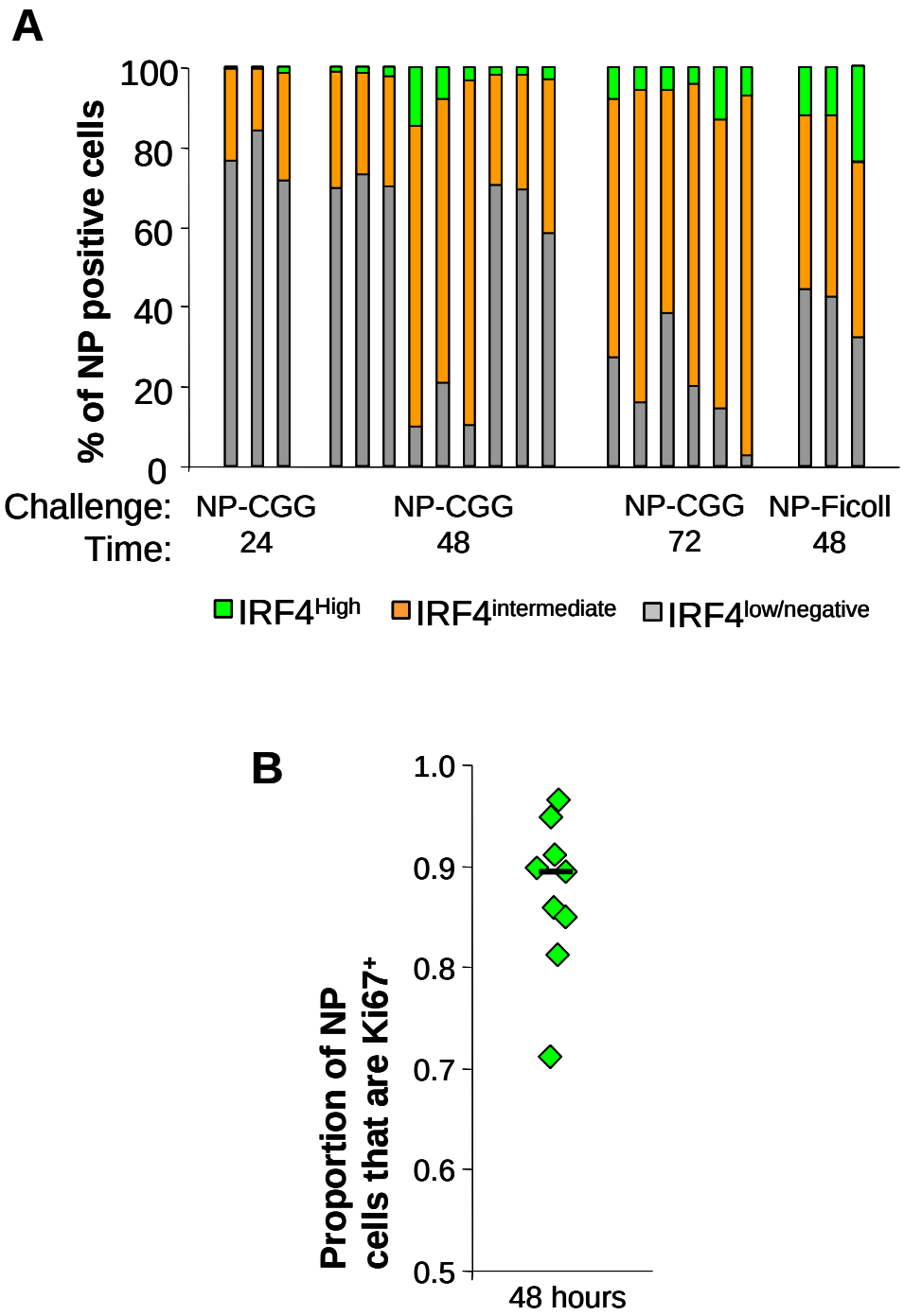


Figure 5.7: Expression of IRF4 reflects the small number of plasmablasts and large numbers of germinal centres produced by the carrier primed response to NP-CGG

**A.** The proportion of NP+ cells that express high, intermediate and low levels of IRF4 protein. At 48 hours a high proportion of cells are $IRF4^{low}$ and very few are $IRF4^{high}$. **B**. The proportion of NP+ cells at 48 hours in the NP-CGG carrier primed response that are Ki67 positive.

The 30 μg NP-Ficoll response in QMxB6 consistently induced a large $B220^{int}$ B blast population. Strikingly this is not induced in carrier primed chimeras receiving 30μg NP-Ficoll or NP-CGG (compare Fig 5.6A with 3.4B). This difference may reflect, at least in part, the difference in number of NP-binding cells in chimeras and QM x B6 mice before immunization. A strong effect of altering NP-specific B cell numbers was noted by Garcia de Vinuesa *et al* (2000) where GC formation in response to NP-Ficoll was seen to fall off when precursor cell numbers were reduced. In this study it was suggested that high NP-specific B cell numbers might lead to stronger BCR signals through cells cross-linking one to the other. On the other hand it might be that cell-cell cross-linking impairs BCR clustering and signalling and that the $B220^{int}$ population are anergic B cells. Further studies are required to probe the significance of the $B220^{int}$ NP-specific cells.

**5.4 The expression of Bcl6 and AID mRNA in germinal centre precursors**

In order to examine the expression of Bcl6, AID, Blimp and IRF4 mRNA in $NP^+$ cells that participate in the carrier primed response to NP-CGG or NP-Ficoll we sorted NP-binding cells from the spleens at 48 and 72 hours after immunization. To prevent contamination from CGG plasmablasts, and because there were too few $NP^+$ $CD138^+$ cells to sort reliably, all the sorts excluded $CD138^+$ cells. The small number of cells in the response meant that the $NP^+$ $CD138^-$ cells were treated as one population and not subdivided into $B220^{high}$ and $B220^{int}$ populations.

As was anticipated, there were NP-binding cells that express both Bcl6 and AID mRNA at 72 hours after NP-CGG administration (Fig 5.8). In contrast to all the immunization protocols studied in the QMxB6 mouse, up to ~10% of the NP-binding cells in the carrier primed response to NP-CGG express Bcl6 and AID mRNA at 48

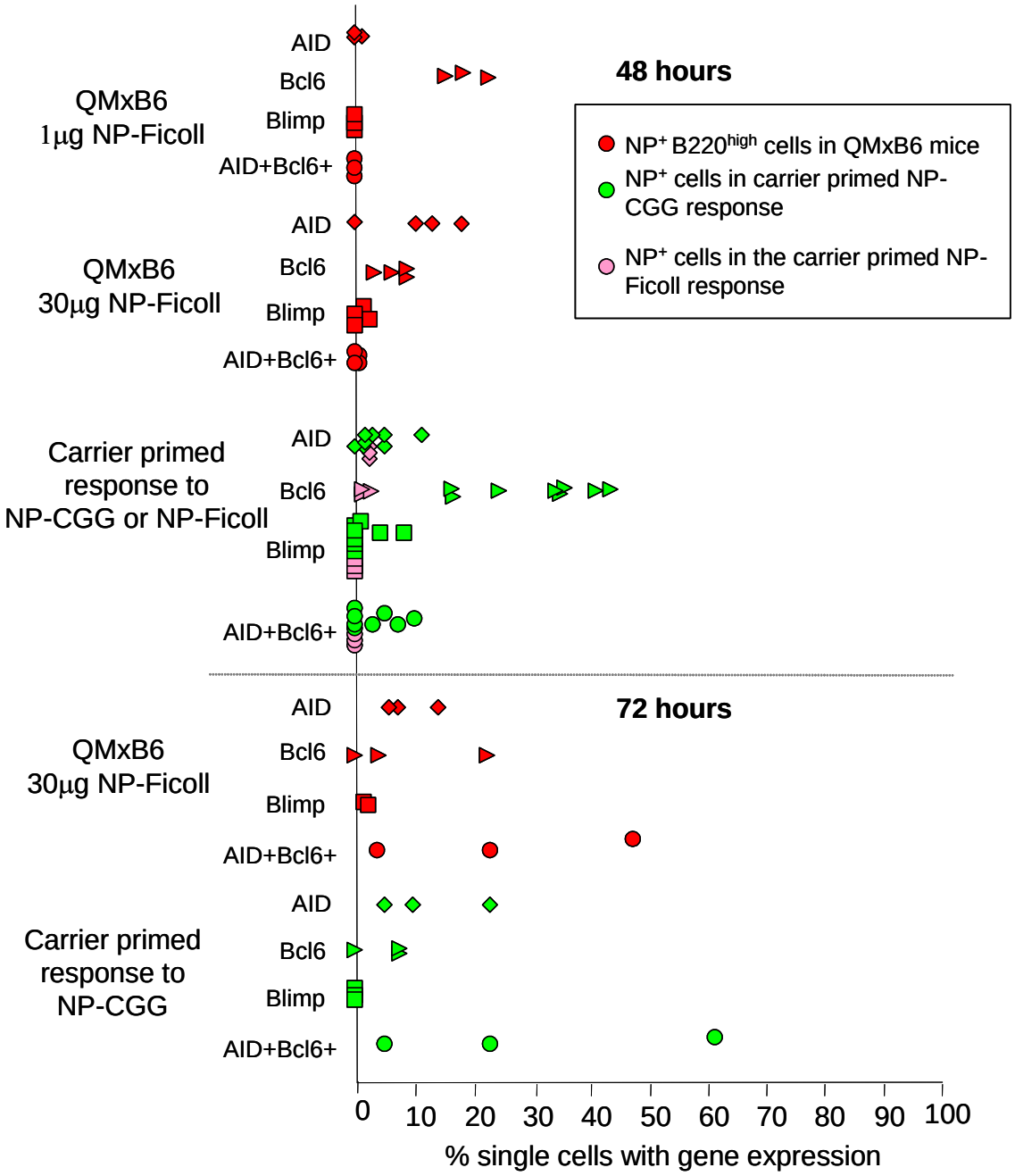


Figure 5.8: A comparison of Bcl6, AID and Blimp1 expression in NP$^+$ cells in the QM x B6 response to 30μg and 1μg NP-Ficoll and the carrier primed NP response to NP-CGG and NP-Ficoll.

The percentage of cells with expression for each gene is indicated for each response at 48 or 72 hours. In the QMx B6 responses B220$^{high}$ are red. In the carrier primed response those responding to NP-CGG are green whilst the response to NP-Ficoll is pink.

hours (Fig. 5.8). Careful scrutiny of the immunohistology on the 4 of 8 spleens taken at 48 hours from which Bcl6/AID mRNA double positive cells had been sorted showed that at best < 1% of the NP-biding cells in the follicles had moved to pre-formed GC. In all of these 48 hour mice the overwhelming majority of the NP-binding cells were focussed towards the outer edge of the follicles. Together these data indicate that at least a proportion of GC precursors in this response co-express AID and Bcl6 mRNA before they enter GC. On the other hand these cells are already in the follicular mantles or among the $IgD^+$ cells in primary follicles. This early expression of the GC B cell phenotype may reflect the availability of T cell help and the formation of GC precursors in this response. Despite the GC bias of the response it is interesting that the percentage of cells that express both AID and Bcl6 mRNA at 72 hours is similar to that of the 30µg NP-Ficoll QMxB6 response (Fig 5.8). The response to NP-Ficoll in carrier primed mice was not tested at 72 hours and so a direct comparison between the two responses can not be made. However, at 48 hours there were no cells that expressed both Bcl6 and AID mRNA after NP-Ficoll challenge in the transferred QM cell response to 30µg NP-Ficoll (Fig. 5.8).

The proportion of $NP^+$ cells that express Bcl6 mRNA but not AID mRNA is ~30% on the second day of the carrier primed response to NP-CGG (Fig. 5.8). This is significantly greater than that seen in the QMxB6 response to 30µg NP-Ficoll (~10%) and much greater than the response of transferred cells to NP-Ficoll (~1.5%). The large proportion of cells that express Bcl6 mRNA may reflect an inhibition of Bcl6 mRNA downregulation, perhaps due to the increase in GC precursors and decrease in plasmablast precursors.

## 5.5 Class switching and AID expression occur in the absence of Bcl6 expression in the $NP^+$ carrier primed response.

In 7 of the 8 mice in the carrier primed response to NP-CGG at 48 hours had a small proportion (median 3%) of $AID^+$,$Bcl6^-$ cells (Fig. 5.8) and in 3 mice tested at 72 hours the proportion of these cells was between 5-23% of NP-binding cells during the third day. Only one of these three mice had appreciable numbers of NP-specific plasmablasts visible on spleen sections. It must remain a matter of speculation whether the $AID^+$, $Bcl6^-$ cells in these 48 and 72 hour mice are pre-plasmablasts, GC precursor cells or cells that will die without further differentiation. Sections from three of the mice at 72 hours that had appreciable, but still low, numbers of plasmablasts were assessed for evidence of CSR and a small proportion of these had switched Ig class (Fig. 5.9). Although one can speculate that GC precursors may undergo CSR there is no obvious way to test this as the first identifiable GC cells are $AID^+$, $Bcl6^+$ cells and the AID expression associated with being a GC cell could theoretically trigger CSR.

## 5.6 Expression of Blimp1 in occasional $CD138^-$ $NP^+$ cells

In all the previous immunization protocols studied single cells expressing Blimp1 were almost exclusively among the $CD138^+$ cells and >90% of $CD138^+$ cells were $Blimp1^+$. This led to the conclusion that Blimp1 expression post dates the expression of CD138. Rare exceptions to this rule are seen among the $CD138^-$ B blasts at 48 hours in the NP-CGG response of carrier primed chimeras. This is particularly surprising as this is the response associated with the smallest number of plasmablasts. The significance of this finding is unclear.

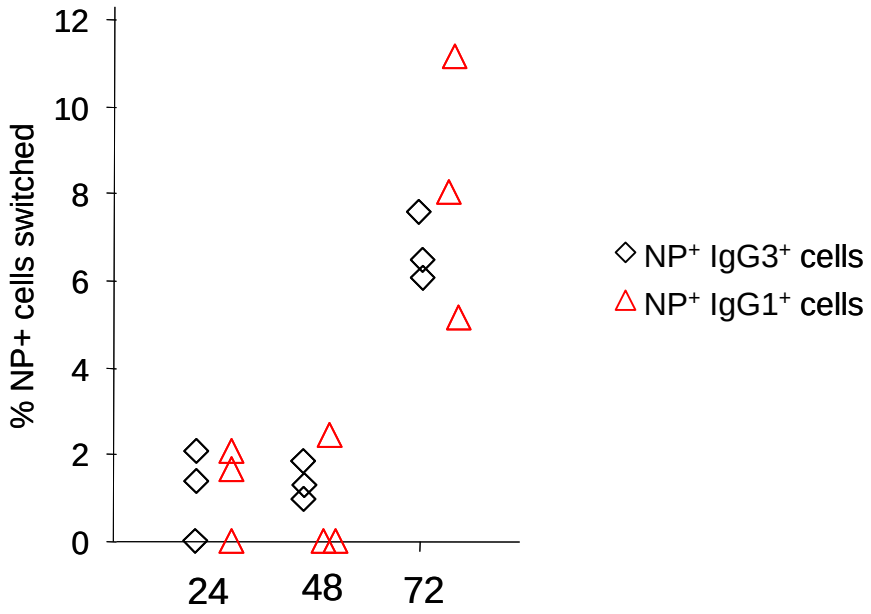


Figure 5.9: Class switch recombination to γ1 and γ3 in $NP^+$ B cells participating in the carrier primed response to NP-CGG

The percentage of $NP^+$ cells that have switched to $IgG_3$ (black diamonds) or $IgG_1$ (red triangles) in the carrier primed response to NP-CGG.

## 5.7 Discussion

*5.7.1. B cells interact with follicular T cells prior to the infiltration of germinal centres in the response to NP-CGG*

It was striking that NP-binding cells participating in the carrier primed NP-CGG response were located in the outer half of the follicle at 48 hours. In the QMxB6 TI responses to NP-Ficoll B cells at 48 hours were seen to be located at the boundary of the T zone and the follicle (Fig 3.2; 4.3; 4.10). Many studies have identified the outer T zone as the location where primed T cells interact with B cells that have taken up antigen (Liu, *et al*, 1991; Toellner, *et al*, 1996; Garside, *et al*, 1998). Indeed, the transferred QM response to NP-Ficoll at 48 hours showed the responding cells at the T zone/follicular boundary (section 5.5). Around 70% of the NP-binding B cells in the outer half of the follicle 48 hours after NP-CGG are in contact with a $CD3^+$ T cell (Fig, 5.5) and 90% of these cells express Ki67 indicating that they are in cell cycle (Fig 5.7). This is prior to the entry of NP-binding cells into GC which only starts during the third day after immunization.

The location of the transferred QM cells in the outer half of the follicle after NP-CGG can not be ascribed to differences in the affinity of the BCR as QM cells were transferred into both the NP-Ficoll and NP-CGG immunized recipients. A difference between the NP-Ficoll and NP-CGG response by transferred QM cells (if we assume that in both cases antigen was not limiting) is that only NP-CGG could be taken up, processes and presented in a way recognised by the primed follicular T cells induced by prior immunization with CGG. Although this would allow cognate interaction between the primed T cells and the B cells presenting CGG peptide it does not explain how these B cells were attracted into the follicles. Clearly BCR

ligation alone is not sufficient to achieve this as both NP-Ficoll and NP-CGG would have cross-linked the BCR of transferred NP-specific B cells. Perhaps the location of the NP-specific cells 24 after immunizing with NP-CGG provides a clue. At this stage the NP-specific cells tended to by sited in the edge of follicles adjacent to bridging channels. Some of these cells were seen to be adjacent to T cells at that time. Could it be that cognate interaction occurring in this T zone follicular boundary area directed the B cells into the outer aspect of the follicles? In my experimental system the majority of the transgenic B cells transferred originated in the MZ. Marginal zone B cells have been shown to provide greater T cell activation than those of follicular origin (Attanavanich and Kearney, 2004). This combination of memory T cells and increased activation may promote pre-GC entry in to the follicle rather than the expected proliferation in the T zone. Migration to the edge of the follicle may have been induced following up regulation of CXCR5 (and down-regulation of CCR7) on the responding T and B cells (Hardtke, *et al*, 2005) permitting movement along the CCL21 gradient established between the follicle and the T zone (Okada, *et al*, 2005). A similar pattern of B cell location, at the outer edge of the follicle, has also been induced by the ablation of dendritic cells prior to virus infection (Scandella, *et al*, 2007). This might indicate a bypass of DC in transferred cell response to NP-CGG or may be a coincidence. Whatever the explanation this novel finding of the early association of antigen-specific CD4 T cells and B cells in the outer part of follicles in carrier primed chimeras deserves further investigation.

*5.7.2. The effect of precursor cell number on B cell migration within the carrier primed NP-CGG response.*

In the course of these carrier primed experiments cell transfers were performed using either $2x10^6$ or $10^5$ $NP^+$ B cells from QM mice. Histological analysis of the NP-CGG response showed that the change in precursor number had no clear influence on the migration of cells within the follicle, or on the formation of GC or plasmablasts (Fig 5.1C, 5.2A and B). However, the three mice examined at 48 hours after NP-CGG that received fewer cells do appear to have an advanced mRNA response to antigen as the proportion of the cells that co-express Bcl6 and AID mRNA and AID mRNA alone is higher in these mice (section 5.4).

That a response with fewer B cells might express differentiation markers before a response with a greater number initially appears strange. Work by Garcia de Vinuesa (2000) has shown that the number of cells that are involved in a TI response can affect the outcome of the response; lower precursor numbers result in fewer GC and plasmablasts. In contrast, the B cell response to a T cell dependant antigen requires that a very small number of specific B cells first bind antigen and then make contact with a specific T cells (Liu, *et al*, 1991; Okada, *et al*, 2005) and the secondary response proceeds with faster kinetics (Liu, *et al*, 1991). In relation to a “normal” secondary TD response my model system has many more precursors. However, as previously stated, the high proportion of Ki67 expression and location next to T cells would suggest that the transfer of a large number of B cells in the response does not overwhelm the T cell help. This does not provide an explanation for the increase in AID mRNA alone or the earlier expression of AID with Bcl6, none the less, the difference exists.

# 6. Conclusions and Future Work

The work describe in this thesis has added to our knowledge of the way B cells are recruited into antibody responses and the stages they go through before either forming germinal centres (GC) or differentiating into plasmablasts. The study also pinpoints where class switch recombination (CSR) occurs during the extrafollicular response. Inevitably the results have identified a number of points that require further study.

## 6.1 The location of B cells 24 hours after antigen engagement is dependent on the type of response induced and the help available

Whilst immunization with NP-Ficoll induced migration of $NP^+$ B cells from the marginal zone (MZ) across the follicle to the T zone/follicular boundary within 24 hours (Fig 3.1, Y. Zhang, *pers. com*), the carrier primed response in chimeric C57Bl6 mice appears to show that after 24 hours the responding $NP^+$ B cells are found at the edge of the follicles where they abut to both the T zone and bridging channels (Fig 5.3). B cells have been reported to migrate to the boundary of the T zone and the follicle in many studies (Vinuesa, *et al*, 2001; Okada, *et al*, 2005; Grey, *et al*, 1984; Liu, *et al*, 1991, Figure 3.1) so why do the $NP^+$ B cells in the T cell dependent response in my studies migrate differently? Marked recirculating B cells transferred into syngeneic rats were found to migrate from the marginal sinus past the edge of follicles adjacent to bridging channels before progressing to the outer T zone (Nieuwenhuis and Ford, 1976). It seems plausible, in the carrier-primed mice used in the studies of the responses to NP-CGG, that primed CGG specific T cells located at the edge of the follicles make cognate interaction with the relatively small numbers of

transferred QM B cells as they pass through this area. The expected result of this would be to stop the progress of the B cells. By contrast the massive numbers of NP-specific B cells that are recruited into the response of QM x B6 are in no position to make cognate interaction with T cells. This is because NP-Ficoll cannot be processed and presented to T cells. Consequently the NP-specific cells in this situation will continue to the outer T zone. Similarly in primary T cell-dependent responses the responding B cells, despite taking up antigen they can process will pass the edge of follicles before follicular T cells have been induced. These T cells only appear from the 3$^{rd}$ day after immunization (Serre, *et al*, 2009).

### *6.1.1 Weak BCR signalling induced by low-dose NP-Ficoll generates some B blasts that die without maturing into either plasmablasts or GC B cells*

The similarity between the QMxB6 response to 1μg NP-Ficoll and that of the btk response to 30μg NP-Ficoll (Vinuesa, *et al*, 2001) must be considered. The response of btk-deficient QM heterozygous male mice to NP-Ficoll induces B cells to migrate to the T zones and enter cell cycle but the cells die apoptosis after around 48 hours without differentiating into plasmablasts or GC. Similarly, the 1μg NP-Ficoll QMxB6 response produced B blasts in the T zone at 48 hours only for the majority to become apoptotic with a small plasmablast response present at 96 hours. The similarities between the two response models suggest that inadequate BCR cross linking after 1μg NP-Ficoll does not instigate sufficient signalling through the BCR to allow differentiation. Whether this relates to failure to activate signalling through btk deserves further study. This might be confirmed through a comparison of the phosophorylation states of btk in both the 30μg and 1μg NP-Ficoll QMxB6 responses to see if it is inadequate in the latter. In the experimental system described by

Vinuesa, *et al*, the block in B cell differentiation induced by NP-Ficoll in Btk-deficient mice is overcome through ligation of CD40. It would be interesting to test whether the B Blast apoptosis associated with 1μg NP-Ficoll response can be prevented in the same way. Array data from both btk QM heterozygotes after 30μg NP-Ficoll with that of the 1μg NP-Ficoll QMxB6 response at 24 might also provide fertile grounds for a comparison between the two responses.

My data suggest that the provision of additional signals (in this case TLR3 ligation by polyI:C) may increase the speed of the response to 30μg NP-Ficoll and induce a greater amount of CSR (Fig 4.9). Toll-like ligand receptor engagement affects both the activated B cells and the milieu (Xu, *et al*, 2008; Xu, *et al*, 2007; Gao, *et al*, 2008). However, whether this is important in the first 24 hours, as a supplement to BCR ligation, or later, in the T zone or follicle, during the response is not covered by my work. This is an important avenue for future study.

### 6.2. Transcriptional changes within the first 24 hours may yet reveal plasmablast and germinal centre precursors.

Within the first 24 hours of the QMxB6 response to 30μg NP-Ficoll, a response that resulted in the production of both germinal centres and plasmablasts, all, or almost all, of the responding B cells upregulated IRF4 protein. They also increased expression of genes involved in DNA repair and the production of antibody while downregulating chemokine receptor mRNA (Figures 3.1; 3.14; 3.15). During the first 24 hours of the response to NP-Ficoll the responding B cells do not proliferate (Hsu, *et al*, 2004; and the present study). This might provide a means of initiating the large transcriptional changes required during differentiation. It is an intriguing possibility that during this long $G_1$ epigenetic changes occur that dictate

subsequent transcriptional and differentiation patterns during the subsequent phase of rapid proliferation. Comparison of epigenetic changes between the responding B cells at 24 hours into the 1μg NP-Ficoll response, with those in the TD carrier primed response to NP-CGG, or in the QMxB6 response to 30μg would be intriguing. Such studies, however, could easily occupy another 3 years' study and were beyond the feasibility of this PhD programme.

Further information on early differentiation changes could be achieved using multiplex single cell RT2-PCR to provide more accurate data on changes within the population sorted (Lambolez, *et al*, 2002). However, this technique does have some limitations. Performing RT2-PCR for more than 3 genes at one time is hindered by mechanical discrepancy between fluorochromes, the competition of competing PCR reactions for reagents and in situ interference between primers and probes. Analysis of the expression of large numbers of genes from a single cell requires a primary phase of amplification without fluorochromes, followed by aliquoting the primary amplificate and then a series of duplex or triplex RT2-PCR. Validating such technology will be a major task.

### 6.3 Expression of AID at 48 hours allows class switch recombination to occur in undifferentiated B blasts

Single cell RT2-PCR studies in QMxB6 mice immunized with NP-Ficoll show that AID is expressed in responding B Blasts at 48 hours independently of GC formation and CD138 or Blimp1 expression. The finding of transcripts of switched heavy-chain among these blasts at this stage of the response confirms that some of these cells have undergone CSR. Furthermore, after administration of 1μg NP-Ficoll in QMxB6 mice, in which there is no CSR, AID expression is negligible (Fig 4.5).

Sciammas, *et al*, (2006) showed that the expression of intermediate levels of IRF4 was necessary for AID to be expressed and CSR to occur. The present studies indicate that this level of expression is not sufficient to induce switching as most, if not all, responding B blasts in the first 2 days of the response express IRF4 at intermediate levels, but only a proportion up-regulate AID (Fig 3.25). Paradoxically the relationship between expression of IRF4 and AID is the inverse of this when the B blasts mature to become GC-founders or plasmablasts. GC founding cells cease to express IRF4 and co-express AID with Bcl6 protein while plasmablasts express high level IRF4, but are universally AID negative.

The addition of polyI:C to 30μg NP-Ficoll increased proportion of switched cells at 96 hours, but this did not yield a greater proportion of B cells expressing AID mRNA on the second day of the response. It is possible that this finding reflects switching occurring earlier when PolyI:C is given. This can be tested by analysing earlier time points within this response. An alternative explanation is that an AID independent CSR pathway is used (Arudchandran, *et al*, 2008).

It is interesting that the location of CSR in the response to antigen does not appear to rely upon a specific location within the white pulp. Forty-eight hours after administration of 30μg NP-Ficoll in the QMxB6 mouse, the majority of cells were in the T zone (Fig 3.2.A). However, the TD carrier primed response to NP-CGG, which was also associated with switching, showed responding cells in the outer half of the follicle at 48 hours (Fig 5.5.A). This discrepancy is likely to reflect the different signalling patterns in these two responses, for in the carrier-primed response to NP-CGG there was evidence of interaction between the responding B cells and T follicular helper cells. The absence of switching after 1μg NP-Ficoll probably reflects

the weak BCR signal delivered (Fig 4.5). A relationship of BCR engagement strength to CSR induction has been reported by Rush, *et al*, (2005) based on studies *in vitro*. However, signals from the microenvironment provided by, for example, dendritic cells, natural killer cells or BAFF and APRIL can induce CSR during the immune response (Gao, *et al*, 2008; Xu, *et al*, 2007; Castigli, *et al*, 2005). Studies in mice have shown the importance of signals through TNF ligand receptors in order to maintain CSR with age (Frasca, *et al*, 2007).

## 6.4 The B220 intermediate population at 48 hours – a meaningless distinction or a relevant phenotype?

Examination of the arrays used in this study show few differences in mRNA expression between the $B220^{high}$ and $B220^{int}$ populations studied at 48 hours (Fig. 3.5). It is tempting to conclude that, prior to differentiation at 72 hours, the responding $NP^{+}$ B cells are largely homogeneous. Nevertheless, the number of any precursors with unique gene expression patterns might be masked in the array analysis. Single cell RT2-PCR of all the QMxB6 responses studied at 48 hours both the $B220^{high}$ and $B220^{int}$ populations showed similar AID, IRF4 and Bcl6 mRNA expression (Figs 3.18, 4.5 and 4.11). Both the $B220^{int}$ and $B220^{high}$ populations contain a small proportion of cells with raised levels of IRF4. This indicates that plasmablasts can form and are forming from both of these subsets. Nevertheless, for most of the cells at 48 hours the above markers assessed at the single cell level fail to provide evidence that the cells or a fraction of the cells is committed to differentiate to either GC founding cells or plasmablasts.

While the $B220^{int}$ population formed 20-40% of the responding B cells in the QMxB6 response to 30μg NP-Ficoll at 48 hours it comprised less than 10% after 1μg

NP-Ficoll, co-immunization with poly I:C and 30μg NP-Ficoll, and during the carrier primed response to NP-CGG (Section 3.4B; Figures 4.4.A; 4.10.A; 5.6.A). The possibility that some of this $B220^{int}$ population have been induced to be anergic deserves consideration. Anergy is reported to occur in B cells that do not receive additional survival signals from T or other accessory cells after BCR engagement (Bretscher and Cohn, 1970; Cook, *et al*, 1998; Goodnow, *et al*, 1989). The phenomena of anergy has been well studied in transgenic B cell mouse models in which a surplus of antigen, either intrinsic to the animal or applied from outside, leads to the development of an anergic B cell population that does not respond to antigen yet remains in circulation (Goodnow, *et al*, 1989; Goodnow, *et al*, 1988; Gauld, *et al*, 2005; Borrero and Clarke, 2002). The QMxB6 30μg NP-Ficoll response mimics these conditions; an excess of antigen is combined with large numbers of responding cells and a lack of second signal. If it is the case that the 30μg NP-Ficoll response induces anergy it is tempting to suggest that the additional TLR signal provided by polyI:C in the co-immunized QMxB6 response is responsible for reduced the size of the $B220^{int}$ population. Equally the carrier primed response provides T cell help in the T zone and subsequently the $B220^{int}$ population was very small (Bretscher and Cohn, 1970). Furthermore, it has been reported that anergic B cells express Cyclin D2 and integrin α4 (Glynne, *et al*, 2000) which were expressed at slightly higher amounts in the $B220^{int}$ population at 48 hours (Fig. 3.15). Another possibility is that the $B220^{int}$ cells are responding B1b cells. In this case the difference in the proportions of $B220^{int}$ cells in the different immunization schedules might reflect a need for a high threshold of BCR cross-linking to activate these cells. Further flow cytometry, single cell RT2-PCR analysis of the $B220^{int}$ population together with functional studies are required to address these possibilities. Cell

transfer into Rag recipient mice would provide a means to phenotype the daughter cells of the $B220^{int}$ population.

### 6.5 The level of expression of IRF4 indicates plasmablast precursors at 48 hours but does not obviously identify germinal centre precursors

Close examination of IRF4 mRNA expression has shown that higher levels of IRF4 mRNA at 48 hours correlate with the presence of plasmablasts at 72 and 96 hours. This, combined with the role of IRF4 in AFC development (Mittrucker, *et al*, 1997; Klein, *et al*, 2006) suggests that these cells are plasmablast precursors. These experiments go some way to confirm the model of B cell activation proposed by Sciammas (2006) whereby higher levels of IRF4 induce plasmablast cell fate. Whilst GC B cells do not express IRF4 (Falini, *et al*, 2000; Fig 3.19.A) it is not certain that low levels of IRF4 mRNA at 48 hours indicate a GC precursor (Fig 4.4B). The distribution of IRF4 expression levels in responding NP-specific cells at 48 hours shows the putative plasmablast precursors with high levels of IRF4 and a Gausian distribution of IRF4 in the remaining cells. It could be that the cells on the left hand tail of this curve are enriched for GC forming cells. Unfortunately as IRF4 is expressed within the cell this could only be addressed by cell sorting and cell transfer unless an internal reporting system for IRF4 expression was developed.

Whether IRF4 levels are responsible for plasmablast and GC precursor B cells, and how this relates to the BCR affinity for the antigen, might be confirmed through the use of a competition model such as that used by Shih, *et al* (2002a). Studies in mice with B cells that either had high or low affinity for NP, such as the B18 high and lo mice used by Shih, have shown that those cells with high affinity for the antigen are preferentially selected to form GC in TD responses, whilst lower

affinity B cells enter the plasmablast pathway (Shih, *et al*, 2002b). This would allow the two pathways to be analysed in TD responses in a way analogous to that achieved in the present thesis using high and low doses of a TI-2 antigen. A comparison of IRF4 mRNA expression in the high with low affinity mice responding to NP-CGG might allow a more detailed examination of the role or IRF4.

How does IRF4 fit into the hierarchy of plasmablast differentiation markers (IRF4, Blimp1 and CD138)? My data suggests that the IRF4 mRNA$^{high}$ cells arise prior to CD138 protein expression. CD138 cells are predominantly Blimp1 mRNA positive and a negligible number of Blimp1 cells are not CD138, consequently it may be stated with some confidence that CD138 expression precedes that of Blimp1 and both are preceded by the upregulation of IRF4 to high levels.

**6.6 Bcl6 mRNA expression is continuous in inactive B cells and does not identify GC precursors or the outcome of the response.**

Bcl6 mRNA is present in ~40% of NP binding B cells in non-immunized QMxB6 mice (Fig 3.18.B). As these B cells do not express Bcl6 protein it raises the question as to why post transcriptional repression occurs in these cells. The decline in Bcl6 mRNA expression as the response progresses (Fig. 3.18) may be as a result of the global action of IRF4 which has been reported to inhibit Bcl6 (Teng, *et al*, 2007; Saito, *et al*, 2007) allowing early plasmablast formation. Further studies are required to identify if there is function significance to those naive B cells that express Bcl6 mRNA and those that do not. Are the Bcl6 mRNA positive cells one of the known subsets of B cells?

The proportion of cells that expressed Bcl6 mRNA at 48 hours after immunization was seen to be largest in the chimeric response to NP-CGG (Fig 5.8)

suggesting a correlation between Bcl6 expression and GC formation later in the response. The known oligoclonality of GC, perhaps makes it unlikely that all the Bcl6 mRNA expressing NP-specific naive cells in QMxB6 mice are committed to germinal centre formation.

In conclusion the studies reported in this thesis have added to our understanding of divergent B cell differentiation during antibody responses. The time when divergent differentiation becomes overt has been defined. It remains to be seen if the level of commitment to GC or plasmablast differentiation is apparent at the molecular level in the first 36 hours of the response. The thesis highlights the importance of single cell analysis to probe for this early heterogeneity. Flow cytometry and immunohistology are powerful tools for studying heterogeneity, but the number of parameters that can be analyzed in single cells by these techniques is limited. In this study RT2-PCR on single cells was also restricted. This technology has the potential to be developed for the analysis of transcription of many more genes within a single cell. As such it may offer the most promising approach for identifying putative key molecular events during the development of heterogeneity in lymphocytes in immune responses. Once identified the functional significance of differential expression will then have to be pursued by other means.

# Appendix

## i. Buffers:

*Tris Buffer pH 7.6*

1.5L Physiological NaCl
1.5L 0.1M HCl
1.0L 0.2M Tris

*Tris Buffer pH 9.2*

As above but pH to 9.2 with HCl

*Peroxidase Substrate*

Dissolve 1 tablet of 3, 3'-diaminobenzidine tetrahydrochloride (DAB) (Sigma, Gillingham, UK) in 15mls of Tris Buffer pH 7.6. Filter to 10mls and add 1 drop of hydrogen peroxide (Sigma).

*Alkaline Phosphate Substrate*

Dissolve 8mgs of levanisole (Sigma) in 10mls of Tris Buffer pH 9.2. In a fume hood dissolve 4mg of NapHal AS-MX-phosphate (Sigma) in 380µl of dimethyl formamide in a glass bottle. Add this to the middle of the levanisole solution. Add 10mg of Fast Blue BB salt (Sigma). Filter.

**ii. Array contents:**

| Gene | Reference | ABI Assay Number |
|---|---|---|
| *Plasma Cell Specific* | | |
| TACI | | Mm00840182_m1 |
| Bcl-xl | | Mm00437783_m1 |
| IL1R1 | | Mm00434237_m1 |
| lL10Rα | | Mm00434151_m1 |
| lL10Rβ | | Mm00434157_m1 |
| c-myc | 2 | Mm00487803_m1 |
| Cyclin D1 | 2 | Mm00432359_m1 |
| Fas | 2 | Mm00433237_m1 |
| IL6R | 2 | Mm00439653_m1 |
| CHOP C/EBP homologous prtn | 2 | Mm00492097_m1 |
| XBP-1 | 2 | Mm00457359_m1 |
| lL4Rα | 3 | Mm00439634_m1 |
| Bob-1 | | Mm00448326_m1 |
| Oct-2 | | Mm00448346_m1 |
| Pu.1 | | Mm00488140_m1 |
| Blk | | Mm00432074_m1 |
| sit | | Mm00450829_g1 |
| Bcl-2 | 4 | Mm00477631_m1 |
| Caspase 6 | 4 | Mm00438053_m1 |
| CD40 | 4 | Mm00441895_m1 |
| CD79α | 4 | Mm00432423_m1 |
| Cyclin D2 | 4 | Mm00438071_m1 |
| PC-1 | 4 | Mm00501097_m1 |
| BAFF rec | | Mm00840578_g1 |
| Blimp-1 | | Mm00476128_m1 |
| J-chain | | Mm00461780_m1 |
| gp130 | | Mm00439668_m1 |
| IL5R | | Mm00434284_m1 |
| GAS6 | 3 | Mm00490378_m1 |

| | | |
|---|---|---|
| ELL2 | 3 | Mm00507237_m1 |
| MAN1A1 | 3 | Mm00484754_m1 |
| IRF4 | 3 | Mm00516431_m1 |
| SSR1 | 3 | Mm00503135_m1 |
| LGALS3 | 3 | Mm00802901_m1 |
| PDXK | 3 | Mm00552031_m1 |
| BCMA | 3 | Mm00495683_m1 |
| DCN 3 | | Mm00514535_m1 |
| KDELR2 | 3 | Mm00482212_m1 |

*Chemokine receptors*

| | | |
|---|---|---|
| CCR1 | | Mm00438260_s1 |
| CCR3 | | Mm00515543_s1 |
| CCR4 | | Mm00438271_m1 |
| CCR7, CD197 | | Mm00432608_m1 |
| CCR8 | | Mm00843415_s1 |
| CCR10 | | Mm00445551_m1 |
| CCR11 | | Mm00521339_m1 |
| CCRL2 | | Mm00516914_g1 |
| CXCR2, IL8Rβ, CD128 | | Mm00438258_m1 |
| CXCR3, CD183 | | Mm00438259_m1 |
| CXCR5 2 | | Mm00432086_m1 |
| CXCR6 | | Mm00472858_m1 |
| CX3CR1 | | Mm00438354_m1 |
| XCR1, GPR5, Ccxcr1 | | Mm00442206_s1 |

*GC specific*

| | | |
|---|---|---|
| GCET1 | | Mm00510959_m1 |
| GCET2 | | Mm00492306_m1 |
| BLNK | | Mm00456139_m1 |
| Bach2 | | Mm00464379_m1 |
| Pax5 | 2 | Mm00435501_m1 |
| Bcl-6 | 2 | Mm00477633_m1 |
| AID | 2 | Mm00507774_m1 |

| Gene | Reference | ABI Assay Number |
|---|---|---|
| CD72 | 2 | Mm00514270_m1 |
| UNG | | Mm00449156_m1 |
| Ku80 | | Mm00550147_m1 |
| Telomerase | | Mm00436931_m1 |
| ARTEMIS | | Mm00455364_m1 |
| XRCC4 | | Mm00459213_m1 |
| DNA-PKcs | | Mm00465092_m1 |
| Rag-2 | | Mm00501300_m1 |
| Human SAK | 1 | Mm00550358_m1 |
| Human CDKN2C | 1 | Mm00483243_m1 |
| Human BRCA-1 | 1 | Mm00515386_m1 |
| Human DNA glycosylase | 1 | Mm00501781_m1 |
| Human DNA ligase I | 1 | Mm00495331_m1 |
| Human CD30L | 1 | Mm00437153_m1 |
| Human CALLA | 1 | Mm00485028_m1 |
| Human RGS13 | 1 | Mm00462629_m1 |
| Human prolyl 4-hydroxylase α (II) subunit | 1 | Mm00477940_m1 |
| Human KIAA0277 | 1 | Mm00616228_m1 |
| Human γ 1 isoform of 61kDa reg. subunit of PP2A | 1 | Mm00442556_m1 |
| Human Cam Kinase I | 1 | Mm00449560_m1 |
| Human RSRFC2 | 1 | Mm00484950_m1 |
| Human AML1 | 1 | Mm00486762_m1 |
| Human: zx55e01.r1 | 1 | Mm00479224_m1 |
| Human Alpha-Catenin-Like Protein | 1 | Mm00490001_m1 |
| Human PGP 9.5 | 1 | Mm00495900_m1 |
| Human mRNA for KIAA0807 protein | 1 | Mm00485291_m1 |
| Human CLI | 1 | Mm00442773_m1 |
| Human cDNA DKFZp586F1018 | 1 | Mm00460563_m1 |
| Human BRCA2 | 1 | Mm00464783_m1 |
| *Both* | | |
| β-actin | | Mm00607939_s1 |
| β-2 microglobulin | | Mm00437762_m1 |

| Gene | Reference | ABI Assay Number |
|---|---|---|
| TLR4 | | Mm00445274_m1 |
| CD20 | | Mm00545909_m1 |
| CD22 | | Mm00515432_m1 |
| CD11α | | Mm00801807_m1 |
| Integrin α4 | | Mm00439770_m1 |

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